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(54) Title: MODULATION OF HEREGULIN AND HER3 INTERACTION

(57) Abstract: The disclosure provided herein identifies and characterizes the domain in HER3 receptor that interacts with heregulin ligand. Typical embodiments of the invention disclosed herein include HER3 variant polypeptides having amino acid sequences which differ from the native HER3 polypeptide sequence and which have altered affinities for heregulin. Also disclosed herein are methods and materials for identifying compounds that bind to the heregulin binding domain in HER3 as well as methods and materials for modulating the interaction between HER3 and heregulin.

## METHODS AND MATERIALS FOR CHARACTERIZING AND MODULATING INTERACTION BETWEEN HEREGULIN AND HER3

### RELATED APPLICATIONS

This application claims priority under Section 119(e) from U.S. Provisional Application Serial No. 60/308,431 filed July 27, 2001, the contents of which are incorporated herein by reference.

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### FIELD OF THE INVENTION

This invention relates generally to HER3 compositions, and to methods of using HER3 compositions to modulate the interaction between heregulin and HER3 and for example, the biological activities of heregulin and HER3. The invention also relates to methods of identifying molecules that bind to the heregulin binding site of HER3. The 10 invention also relates to methods for *in vitro*, *in situ*, and/or *in vivo* diagnosis and/or treatment of mammalian cells or pathological conditions associated with HER3 and heregulin.

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### BACKGROUND OF THE INVENTION

Cancers are the second most prevalent cause of death in the United States, causing 450,000 deaths per year. One in three Americans will develop cancer, and one in five will die of cancer. While substantial progress has been made in identifying some of the likely environmental and hereditary causes of cancer, there is a need for substantial improvement in the diagnosis and therapy for cancer and related diseases and disorders.

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A number of so-called cancer genes, i.e., genes that have been implicated in the etiology of cancer, have been identified in connection with hereditary forms of cancer and in a large number of well-studied tumor cells. Cancer genes are broadly classified into "oncogenes" which, when activated, promote tumorigenesis, and "tumor suppressor genes" which, when damaged, fail to suppress tumorigenesis. While these classifications 25 provide a useful method for conceptualizing tumorigenesis, it is also possible that a particular gene may play differing roles depending upon the particular allelic form of that

gene, its regulatory elements, the genetic background and the tissue environment in which it is operating. Human epidermal growth factor receptor 3 is one of these genes implicated in the etiology of cancer (see, e.g. Munster et al., Cancer Res. 2002 Jun 1;62(11):3132-7; Menard et al. J Cell Physiol 2000 Feb;182(2):150-62; Basso et al., 5 Oncogene 2002 Feb 14;21(8):1159-66; and Yarden Oncology 2001;61 Suppl 2:1-13).

Human epidermal growth factor receptor 3 (HER3) (see, e.g. Kraus et al., (1989) Proc. Natl. Acad. Sci. U. S. A. 86, 9193-9197) is a member of the type I receptor tyrosine kinase (RTK) family, which also includes EGFR, HER2/*neu*, and HER4 (see, e.g. Ullrich et al., (1984) Nature 309, 418-425; Schechter et al., (1985) Science 229, 976-978; 10 Plowman et al., (1993) Proc. Natl. Acad. Sci. U. S. A. 90, 1746-1750). HER3 forms heterodimers with other members of the type I RTK family, including the HER2/*neu* receptor (see, e.g. Sliwkowski et al., (1994) J. Biol. Chem. 269, 14661-14665; Fitzpatrick et al., (1998) FEBS Lett. 431, 102-106; Heldin, C. H. (1995) Cell 80, 213-223; Tzahar et al., (1997) EMBO J. 16, 938-4950). The HER2/*neu* receptor is amplified and overexpressed in 25-30% of human breast and 8-11% off human ovarian cancers. This 15 overexpression correlates with increased morbidity and mortality, and there is evidence that the overexpressed HER2 receptor leads to aggressive malignancies (see; e.g. Slamon et al., (1987) Science 235, 177-182; Slamon et al., (1989) Science 244, 707-712; Plowman et al., (1993) Nature 366, 473-475; Dougall et al., (1996) DNA Cell Biol. 15, 31-40).

20 Cells expressing only HER2 receptors alone and not other members of the EGFR family fail to bind heregulin, but HER2/neu has high tyrosine kinase activity. HER3 is a kinase defective receptor (see, e.g. Guy et al. (1994) Proc Natl Acad Sci U S A 91(17), 8132-6), but has binding affinity for heregulin (see, e.g. Carraway et al. (1994) J Biol Chem 269(19), 14303-6). The HER2/HER3 heterodimer forms a high affinity 25 heregulin receptor with tyrosine kinase activity. Heregulin binding to cells that display the HER2/HER3 heterodimer causes a mitogenic response both *in vitro* and *in vivo*, so understanding this interaction is of medical importance (see, e.g. Aguilar et al., (1999) Oncogene 18, 6050-6062; Sliwkowski et al. (1994) J Biol Chem 269(20), 14661-5; Heldin,

C. H. (1995) *Cell* 80(2), 213-23; Tzahar et al. (1997) *Embo J* 16(16), 4938-50). Alternate transcripts of HER3 isolated from an ovarian carcinoma-derived cell line have been identified which encode truncated forms of the extracellular domain of HER3, including three clones where the protein products were soluble secreted proteins (see, e.g. Lee, H., 5 and Maihle, N. J. (1998) *Oncogene* 16(25), 3243-52). A naturally occurring secreted form of HER3 has been found to inhibit heregulin-stimulated activation of HER3 (see, e.g. Lee et al. (2001) *Cancer Res* 61(11), 4467-73). This provides evidence that HER3 could be an important target in breast cancer therapy.

Type I receptor tyrosine kinases typically contain four extracellular domains, a 10 single hydrophobic transmembrane segment, and a cytoplasmic tyrosine kinase domain (see, e.g. Ullrich, A., and Schlessinger, J. (1990) *Cell* 61, 203-212). HER2/*neu* is a very active tyrosine kinase, but cells expressing HER2/*neu* alone, and not other members of the EGFR family, fail to bind heregulin. Conversely, the HER3 receptor binds heregulin but has low tyrosine kinase activity (see, e.g. Guy et al., (1994) *Proc. Natl. Acad. Sci. U. S. 15 A.* 91, 8132-8136; Carraway et al., (1994) *J. Biol. Chem.* 269, 14303-14306). As noted above, the HER2/HER3 heterodimer is a high affinity heregulin binding complex with signaling activity through the HER-2 kinase domain. To date, the domains of HER3 involved in ligand binding and heterodimerization have not been identified.

Thus far, the high carbohydrate content (see, e.g. Horan et al., (1995) *J. Biol. 20 Chem.* 270, 24604-24608) and the relatively large size (~180 kDa) of the receptors in the EGFR family have frustrated structural analysis by x-ray crystallography and NMR, so other methods have been sought to illuminate the structure and function of HER3. The extracellular domains (ECDs) of the type I RTKs have been divided into four domains: I, II, III, and IV, based on sequence analysis (see, e.g. Yarden, Y., and Ullrich, A. (1988) 25 *Annu. Rev. Biochem.* 57, 443-478). Domains II and IV are cysteine-rich and are similar in sequence. Domains I and III also have sequence similarity (see, e.g. Yarden, Y., and Ullrich, A. (1988) *Annu. Rev. Biochem.* 57, 443-478; Lax et al., (1988) *Mol. Cell. Biol.* 8, 1970-1978). Little is known about the specific function of each domain except in EGFR,

where several lines of evidence provide evidence that the major determinants for EGF binding lie in domain III. These lines of evidence include the following: 1) the exchange of domain III in chicken EGFR for domain III from human EGFR confers binding of human EGF (see, e.g. Lax et al., (1989) EMBO J. 8, 421-427; Lax et al., (1991) Cell Regul. 2, 337-345); 2) monoclonal antibodies that recognize residues in domain III prevent EGF binding to EGFR (see, e.g. Wu et al., (1989) J. Biol. Chem. 264, 17469-17475); 3) cross-linking of EGF to EGFR identified residues in domain III (see, e.g. Summerfield et al., (1996) J. Biol. Chem. 271, 19656-19659; Wu et al., (1990) Proc. Natl. Acad. Sci. U. S. A. 87, 3151-3155); and 4) limited proteolysis of the ECD of EGFR produced a fragment that encompassed domain III, which bound transforming growth factor, with the observation that binding could be enhanced by including portions of domain IV (see, e.g. Kohda et al., (1993) J. Biol. Chem. 268, 1976-1981). Additional studies from cross-linking experiments indicated that bound EGF is also close to tyrosine 101 in domain I of murine EGFR (see, e.g. Woltjer et al., (1992) Proc. Natl. Acad. Sci. U. S. A. 89, 7801-7805). Taken together, these experimental results provide evidence that domain I and III are close to the ligand-binding region in EGFR and that domain III contributes most of the binding. HER3 and EGFR have relatively high sequence identity (45% identity in the ECD) and belong to the same family of type 1 tyrosine kinase receptors; however, they bind a different subset of ligands and differ in preference for heterodimerization *versus* homodimerization (see, e.g. Tzahar et al., (1997) EMBO J. 16, 938-4950; Lemmon et al., (1997) EMBO J. 16, 281-294; Huang et al., (1998) Biochem. J. 331, 113-119; Alimandi et al., (1995) Oncogene 10, 1813-1821).

While the existing art provides a limited understanding of the structure of HER3 and the interaction between heregulin and HER3, this art does not delineate the domains in HER3 responsible for interacting with heregulin. Consequently there is a need in the art for the identification and characterization of the domains in HER3 involved in this interaction so that methods and materials for modulating this interaction can be generated. The disclosure provided herein meets this need.

SUMMARY OF THE INVENTION

- The disclosure provided herein identifies and characterizes the domains in the HER3 receptor that interact with the heregulin ligand. In this context, the present
- 5 invention provides antagonists or agonists of the HER3 receptor such as soluble HER3 variants and methods for using them. The antagonists and agonists described herein find utility for, among other things, *in vitro*, *in situ*, or *in vivo* diagnosis or treatment of mammalian cells or pathological conditions associated with the aberrant expression of heregulin and/or HER3.
- 10 A preferred embodiment of the invention is a HER3 variant polypeptide comprising an amino acid sequence which differs from the native sequence HER3 polypeptide sequence of SEQ ID NO: 2 and has one or more of amino acid substitutions at the following residue position(s) in SEQ ID NO: 2: E43; N44; K51; E64; V66; and V110. Another embodiment of the invention is a non-naturally occurring
- 15 HER3 variant polypeptide consisting essentially of amino acid 20 to amino acid 329 of HER3 polypeptide sequence of SEQ ID NO: 2; wherein the HER3 variant polypeptide specifically binds to the heregulin polypeptide of SEQ ID NO: 4 and exhibits an impaired ability to interact with the HER2 polypeptide of SEQ ID NO: 6.

Related embodiments of this invention include an isolated nucleic acid

20 comprising a nucleotide sequence encoding these HER3 variants, vectors comprising nucleic acids encoding these variants and host cells (e.g. *E. coli*) containing these vectors. Another related embodiment of the invention is a method of making HER3 variant polypeptide by providing a host cell comprising a vector encoding a HER3 variant, providing culture media, culturing the host cell in the culture media under conditions

25 sufficient to express the HER3 variant polypeptide, recovering the HER3 variant polypeptide from the host cell or culture media, and then purifying the HER3 variant polypeptide. Optionally the HER3 variant polypeptide is conjugated or linked to one or more polyol groups.

The invention also provides methods of conducting screening assays to identify candidate molecules, such as small molecule compounds, polypeptides or antibodies, which act as agonists or antagonists with respect to the interaction between heregulin and HER3. One embodiment of the invention is a method of identifying a compound 5 which specifically binds the heregulin binding domain in a HER3 variant polypeptide comprising amino acid 20 to amino acid 329 of HER3 polypeptide sequence of SEQ ID NO: 2 comprising contacting the HER3 variant polypeptide with a test compound under conditions favorable to binding and determining whether the test compound specifically binds to the HER3 variant polypeptide such that a compound which binds to the HER3 10 variant polypeptide can be identified. Optionally this method can further comprise determining whether the test compound inhibits the heregulin induced tyrosine kinase activity by contacting mammalian cells that express HER3 receptor with heregulin in the presence and absence of the test compound and then monitoring the mammalian cells for the tyrosine kinase activity associated with the HER2/HER3 polypeptide complex, 15 wherein an inhibition in tyrosine kinase activity in the presence of the test compound as compared to the absence of the test compound identifies the test compound as an inhibitor of heregulin induced tyrosine kinase activity. Alternatively, the method can further comprise determining whether the test compound enhances the heregulin induced tyrosine kinase activity by contacting mammalian cells that express HER3 20 receptor with heregulin in the presence and absence of the test compound and then monitoring the mammalian cells for the tyrosine kinase activity associated with the HER3 polypeptide, wherein an increase in tyrosine kinase activity in the presence of the test compound as compared to the absence of the test compound identifies the test compound as an enhancer of heregulin induced tyrosine kinase activity.

25 Yet another embodiment of the invention is a method of determining whether a test compound modulates the interaction between heregulin polypeptide of SEQ ID NO: 2 and a HER3 variant polypeptide comprising amino acid 20 to amino acid 329 of SEQ ID NO: 2 by contacting the HER3 variant polypeptide with a test compound under

conditions favorable to binding, contacting the HER3 variant polypeptide with heregulin under conditions favorable to binding and then comparing the binding interaction between the HER3 variant polypeptide and heregulin with the binding interaction between HER3 variant polypeptide and heregulin in the absence of the test compound 5 such that a compound which modulates the interaction between heregulin and the HER3 variant polypeptide can be identified.

Another embodiment of the invention is a method of inhibiting the interaction between a heregulin polypeptide having the sequence shown in SEQ ID NO: 4 and HER3 polypeptide having the sequence shown in SEQ ID NO: 2 comprising exposing 10 the heregulin polypeptide to a non-naturally occurring HER3 variant polypeptide comprising domains I and II of the HER3 polypeptide sequence of SEQ ID NO: 2; wherein the HER3 variant polypeptide specifically binds to the heregulin polypeptide of SEQ ID NO: 4; and exhibits an impaired ability to interact with the HER2 polypeptide of SEQ ID NO: 6. In preferred embodiments of the invention, the HER3 variant 15 polypeptide has an amino acid substitution V110 in SEQ ID NO: 2.

Preferred embodiments of the invention also include anti-HER3 antibodies which are capable of specifically binding to the heregulin binding domain. In a preferred embodiment, the invention provides antibodies which specifically bind to a HER3 polypeptide and inhibit the binding of heregulin ligand to the HER3 receptor. 20 Optionally, the antibodies are monoclonal antibodies.

In one embodiment of the invention, there are provided methods for the use of heregulin antagonists to block or neutralize the interaction between heregulin and HER3. For example, the invention provides a method comprising exposing a mammalian cell, such as an ovarian or breast cell, to one or more heregulin antagonists in an amount 25 effective to decrease, neutralize or block activity of the heregulin ligand. The cell may be in cell culture or in a mammal, e.g. a mammal suffering from, for instance, cancer. Thus, the invention includes a method for treating a mammal suffering from a pathological

condition such as cancer comprising administering an effective amount of one or more heregulin antagonists, as disclosed herein.

In addition, the invention provides methods of using HER3 agonists (e.g. anti-HER3 agonist antibodies which target the heregulin binding domain) to, for instance, 5 stimulate or activate HER3 receptor. Such methods will be useful in treating pathological conditions characterized by or associated with insufficient HER3 expression or activity. The HER3 agonists typically comprise agonistic anti-HER3 antibodies or HER3 variant polypeptides. The agonistic activity of such HER3 agonists may comprise enhancing the activity of a native ligand for HER3 or activity which is the same as or 10 substantially the same as (i.e., mimics) the activity of a native ligand for HER3. Typical methods of the invention include methods to treat pathological conditions or diseases in mammals associated with or resulting from increased or enhanced HER3 or heregulin expression and/or activity. In the methods of treatment, HER3 variant polypeptides may be administered which preferably block or reduce the respective receptor binding or 15 activation by heregulin.

The invention also provides compositions which comprise one or more HER3 or heregulin agonists or antagonists. Optionally, the compositions of the invention will include pharmaceutically acceptable carriers or diluents. Preferably, the compositions will include one or more HER3 or heregulin antagonists or agonists in an amount which 20 is therapeutically effective to treat a pathological condition or disease. Optionally, the compositions will include one or more HER3 agonists in an amount which is therapeutically effective to stimulate signal transduction by HER3.

The invention also provides articles of manufacture and kits which include one or more HER3 or heregulin antagonists or agonists.

BRIEF DESCRIPTION OF THE DRAWINGS

The file of this patent contains at least one drawing executed in color. Copies of this patent with color drawing(s) will be provided by the patent and Trademark Office upon request and payment of the necessary fee.

5       **Fig. 1.** *A*, limited proteolysis of HER3-ECD<sup>I-IV</sup> generates two detectable fragments, which remain associated under oxidizing conditions. HER3-ECD<sup>I-IV</sup> was digested in the presence of trypsin (T) and analyzed in the Phast gel in the presence of the reducing agent  $\beta$ -ME. The proteolytic digest of HER3-ECD<sup>I-IV</sup> was analyzed on an SDS-PAGE Phast gel (10-15%) in the absence (lane 4) and the presence (lane 3) of the  
10 reducing agent  $\beta$ -ME. Nonproteolyzed HER3-ECD<sup>I-IV</sup> in the presence of  $\beta$ -ME is shown in lane 2. The two fragments in proteolyzed HER3-ECD<sup>I-IV</sup> remain linked by one or more disulfide bridges. *B*, a V5 antibody protects HER3-ECD<sup>I-IV</sup> from cleavage at the C terminus. When HER3-ECD<sup>I-IV</sup> is proteolyzed in the absence of the V5 antibody, the C terminus is digested and could not be visualized when probed for the V5 epitope. This  
15 gel shows HER3-ECD<sup>I-IV</sup> proteolyzed for the time indicated in the presence of an antibody against the V5 epitope tag, analyzed on a 4-15% polyacrylamide gradient gel, and transferred to a polyvinylidene difluoride membrane. The C-terminal fragment was identified by Western blotting using an antibody against the C-terminal V5 epitope. The V5 antibody protects the C terminus against proteolytic cleavage and shows that  
20 fragment 2 is the C-terminal fragment.

25       **Fig. 2.** *A*, nonproteolyzed HER3-ECD<sup>I-IV</sup> binds hrg, but proteolyzed HER3-ECD<sup>I-IV</sup> does not. The presence (+) or absence (-) of trypsin in the proteolytic digest is indicated above the lanes. HER3-ECD<sup>I-IV</sup> was analyzed on a native Phast gel (10-15%) in the absence (lane 1) and presence (lane 3) of equimolar hrg. Proteolyzed HER3-ECD<sup>I-IV</sup> was also analyzed in the absence (lane 2) and presence (lane 4) of hrg. Formation of a hrg-HER3-ECD<sup>I-IV</sup> complex is observed with the nonproteolyzed HER3-ECD<sup>I-IV</sup> but not with the proteolyzed HER3-ECD<sup>I-IV</sup>. *B*, nonproteolyzed HER3-ECD<sup>I-IV</sup> is eluted by hrg in a pull-down assay, but proteolyzed HER3-ECD<sup>I-IV</sup> was not. HER3-ECD<sup>I-IV</sup> was

applied untreated (-) or digested in the absence (T) or presence of hrg (T + h) to agarose-bound hrg. HER3-ECD<sup>I-IV</sup> was detected with an antibody with an epitope in fragment 1. HER3-ECD<sup>I-IV</sup> was bound to immobilized hrg and could be eluted with free hrg (lane 3) and detergent (lane 6), but proteolyzed HER3-ECD<sup>I-IV</sup> could not be eluted with hrg (lane 4) or detergent (lanes 7). HER3-ECD<sup>I-IV</sup> that was protected by hrg during proteolytic digestion could be recovered by both hrg (lane 5) and detergent (lane 8). Trypsin that was treated with phenylmethylsulfonyl fluoride prior to addition to the digestion reaction (\*) did abolish hrg binding by HER3-ECD<sup>I-IV</sup>, which could then be recovered by hrg (lane 9). This demonstrates that proteolyzed HER3-ECD<sup>I-IV</sup> does not bind hrg.

Fig. 3. *A*, limited proteolysis of HER3-ECD<sup>I-IV</sup> in the presence of hrg generates a different cleavage pattern than in the absence of hrg when analyzed on an SDS-PAGE Phast gel (10-15%) under reducing conditions. HER3-ECD<sup>I-IV</sup> was proteolyzed in the absence of ligand (-) and in the presence of hrg, NA<sub>5</sub>-hrg, and EGF, as indicated above the lanes. Limited proteolysis of HER3-ECD<sup>I-IV</sup> generates two fragments: one at an apparent molecular mass of 24.2 kDa (fragment 1) and another at 54.1 kDa (fragment 2). In the presence of NA<sub>5</sub>-hrg (lane 4) and EGF (lane 5), fragment 1 appears to be identical to fragment 1 in the absence of hrg (lane 2), whereas in the presence of wild type hrg (lane 3), fragment 1 appears to have a slightly higher molecular mass. The digest of HER3-ECD<sup>I-IV</sup> in the presence of a binding-competent hrg generates a different cleavage pattern than in its absence. Apparently, the cleavage that generates fragment 1 can be protected by hrg and is due to a specific interaction between hrg and HER3-ECD<sup>I-IV</sup>. *B*, the minimum amount of hrg required to produce a change in the molecular weight of fragment 1 in the SDS gel was determined by varying the molar ratio of hrg to HER3-ECD<sup>I-IV</sup>. The ratio of hrg to HER3-ECD<sup>I-IV</sup> was varied from 2.0 to 0 (lanes 2-8), as indicated above the lanes. A change in the molecular weight of fragment 1 was observed when the molar ratio of hrg to HER3-ECD<sup>I-IV</sup> was between

0.9:1 and 1:1. Complete protection of HER3-ECD<sup>I-IV</sup> by hrg was observed at an approximately 1:1 ratio of hrg to HER3-ECD<sup>I-IV</sup>.

**Fig. 4.** Domain organization and proteolytic cleavage sites of HER3-ECD<sup>I-IV</sup>. The extracellular region of HER3 contains domains I, II, III, and IV. Sequence positions 5 are indicated at the start of each domain. The beginning of HER3-ECD<sup>I-IV</sup> at position 20 refers to the start of mature HER3-ECD<sup>I-IV</sup> after cleavage of the leader sequence. The various digests that were analyzed are shown to the left: the digest of HER3-ECD<sup>I-IV</sup> in the presence (+) and absence (-) of hrg and mass spectrometry of the oxidized (-βME) and reduced (+βME) forms of the digest. The position of the cleavage site as determined 10 by N-terminal sequencing is indicated at the start of each fragment. Fragment 1 is indicated by a gray bar, and fragment 2 is represented by a black bar. Numbers in brackets are mass estimates in kDa as determined by mass spectrometry. S-S indicates that the two fragments are held together by a disulfide bond. The exact position of the cleavage at the C terminus is unknown, and this is indicated by a dashed bar. The 15 postulated mass of the missing fragments is indicated in brackets below the fragments with dashed lines. In summary, hrg protects HER3-ECD from cleavage at position 50 but not at any other site.

**Fig. 5.** HER3-ECD<sup>I-IV</sup> binds hrg but not EGF or the binding-deficient NA<sub>5</sub>-hrg. HER3-ECD<sup>I-IV</sup> was analyzed on a native Phast gel (10-15%) in the absence of hrg 20 (lane 1) and in the presence of hrg (lane 2), NA<sub>5</sub>-hrg (lane 3), and EGF (lane 4). The ligands were used at a 1:1 ratio of hrg to HER3-ECD<sup>I-IV</sup>. A complete shift is observed in the presence of hrg, and no shift is apparent in NA<sub>5</sub>-hrg or EGF.

**Fig. 6.** Characterization of recombinant HER3-ECD<sup>I-II</sup> by SDS-PAGE analysis, mass spectrometry, light scattering, and equilibrium centrifugation. *A*, recombinant HER3-ECD<sup>I-II</sup> is pure as judged by SDS-PAGE analysis. HER3-ECD<sup>I-II</sup> was expressed in *Drosophila* S2 cells and purified from the medium. The molecular mass 25 appears to be somewhat less than 40 kDa. *B*, the molecular mass of HER3-ECD<sup>I-II</sup> is between 38.5 and 40 kDa as determined by MALDI mass spectrometry, compared with a

theoretical molecular mass of 37.7 kDa. This provide evidences that HER3-ECD<sup>I-II</sup> has a carbohydrate content between 3.3 and 5.7% by weight. *C*, recombinant HER3-ECD<sup>I-II</sup> is a monomer as determined by size exclusion chromatography followed by multiangle light scattering. Recombinant HER3-ECD<sup>I-II</sup> eluted in a single peak (black line) with a molecular mass of ~34 kDa (S.E. = ± 4%) (black circles). The addition of a 1.3-fold molar excess of hrg caused a slight shift in the peak (gray line). The shifted peak has a molecular mass of 35 kDa (S.E. = ±3%) (gray squares). Recombinant HER3-ECD<sup>I-II</sup> is a monomer and remains a monomer in the presence of hrg. *D*, recombinant HER3-ECD<sup>I-II</sup> is a monomer, as determined by ultracentrifugation at three different concentrations and three different speeds. The average molecular mass of HER3-ECD<sup>I-II</sup> is 35.0 kDa (S.E. = ±0.6%), which corresponds to monomeric HER3-ECD<sup>I-II</sup>.

**Fig. 7.** Recombinant HER3-ECD<sup>I-II</sup> binds hrg. *A*, HER3-ECD<sup>I-II</sup> was analyzed in a pull-down assay in which S-tagged hrg was immobilized on S-protein resin (lanes 3-5) and could be specifically eluted with 1 μM hrg (lane 4) but not PBS (lane 3) or 1 μM EGF (lane 5). The fragment showed little nonspecific binding to S-protein resin without immobilized S-tagged hrg (lanes 1 and 2) when eluted with PBS (lane 1) or 1 μM hrg (lane 2). This shows that domains I and II of HER3-ECD<sup>I-IV</sup> are involved in hrg binding. *B*, recombinant HER3-ECD<sup>I-II</sup> has a K<sub>d</sub> of 68 nM as determined by SPR (BIAcore). HER3-ECD<sup>I-II</sup> showed binding to immobilized trx-hrg with a calculated equilibrium dissociation constant of 68 nM using the three different concentrations of HER3-ECD<sup>I-II</sup> indicated. This shows that HER3-ECD<sup>I-II</sup> binds hrg.

**Fig. 8.** Sequence alignment of domain I of HER3-ECD<sup>I-IV</sup>, IR, and IGF-1R indicates the position of the proteolysis site (▼) in domain I of HER3-ECD<sup>I-IV</sup> relative to mutations (\*) that decrease ligand binding in the IR. A naturally occurring mutation that decreases binding in IR is indicated by an open circle. The "hormone binding footprint" in the IGF-1R structure (43) is indicated by shaded areas in the sequence. This alignment shows that the proteolysis site HER3-ECD<sup>I-IV</sup> that is protected by hrg corresponds to the analogous putative ligand-binding region in domain L1 in IGF-1R.

**Fig. 9.** Structural similarity of IGF-1R to HER3-ECD<sup>I-IV</sup>. A, space-filling model of domains I and II of the structure of IGF-1R (43) showing the positions of the proteolysis sites (red) observed for the HER3-ECD<sup>I-IV</sup> mapped to the IGF-1R structure. The position of mutations (dark blue) that decrease ligand binding in IR are also mapped to a binding footprint in the IGF-1R structure. Domain L1 is light blue, and L2 is lavender in IGF-1R (domains I and III in HER3-ECD<sup>I-IV</sup>), and domain S1 (domain II in HER3-ECD<sup>I-IV</sup>) is green. 1740 Å<sup>2</sup> of accessible surface area in domain L1 are buried by domain S1 in IGF-1R. The cleavage site at position 50 in HER3-ECD<sup>I-IV</sup> that is protected by hrg (position 18 in IGF-1R) lies on the same face of domain L1 in IGF-1R and is 11 Å away from residue 10 in the binding footprint of IR/IGF-1R. The unprotected cleavage site at position 270 in HER3-ECDI-IV (position 237 in IGF-1R) lies in domain II, is on the opposite side as the binding footprint, and is 40 Å away from the binding footprint. Residue numbers are shown in the binding footprint. The cleavage site that is protected by hrg in HER3-ECD<sup>I-IV</sup> is near the binding footprint in domain L1 of IGF-1R. B, backbone representation of domain II of IGF-1R showing the position of the proteolysis site at position 270 in HER3-ECD<sup>I-IV</sup> (red cpk) (position 237 in IGF-1R) superimposed on the IGF-1R structure. The chain remains connected by a disulfide (red ball and stick) when cleavage occurs at position 270. The disulfide bridge connecting fragments I and II of HER3-ECD<sup>I-IV</sup> is consistent with a structural model based on IR/IGF-1R.

**Fig. 10.** A, HER3<sup>I-II</sup> and HER3<sup>I-II E64A</sup> were analyzed in a "pull down assay" in which S-tagged hrg was immobilized on S-protein resin (lanes 3-6) and HER3<sup>I-II</sup> could be specifically eluted with 1 µM hrg and detergent (lane 3 and 5), but HER3<sup>I-II E64A</sup> was not eluted by either 1 µM hrg or detergent (lane 4 and 6). Both HER3<sup>I-II</sup> and HER3<sup>I-II E64A</sup> showed little non-specific binding to S-protein resin without immobilized S-tagged hrg (lanes 1-2). This shows that HER3<sup>I-II E64A</sup> does not bind hrg. B, Recombinant HER3<sup>I-II E64A</sup> had no detectable binding greater than 500 nM to the immobilized hrg on the BIAcore chip while HER3<sup>I-II</sup> showed binding to immobilized trx-hrg with a

calculated equilibrium dissociation constant of 68 nM. This shows that HER3<sup>I-II</sup>E64A does not bind hrg.

Fig. 11. Characterization of recombinant HER3<sup>I-II</sup>E64A by mass spectrometry and circular dichroism. A, the molecular mass of HER3<sup>I-II</sup>E64A is between 38.4 and 40.6 kDa as determined by MALDI mass spectrometry, which is comparable to the molecular mass of HER3<sup>I-II</sup> (38.5 and 40 kDa). This provides evidence that the polypeptide chain of HER3<sup>I-II</sup>E64A is intact. B, the CD spectra of HER3<sup>I-II</sup> and HER3<sup>I-II</sup>E64A were essentially identical between 210 nM and 260 nM provide evidencing that they have the same secondary structure.

Fig. 12. A, HER3<sup>I-IV</sup> binds hrg, but HER3<sup>I-IV</sup>E64A does not. HER3<sup>I-IV</sup> and the HER3<sup>I-IV</sup> mutants were analyzed on a native Phast gel (10-15%) in the absence (-) and presence (+) of equimolar hrg. The HER3<sup>I-IV</sup>E64A was also analyzed in the absence (lane 2) and presence (lane 4) of hrg. Formation of a hrg-HER3<sup>I-IV</sup> complex is observed with HER3<sup>I-IV</sup>, but not with HER3<sup>I-II</sup>E64A. B, HER3<sup>I-IV</sup> and HER3<sup>I-IV</sup>E64A were analyzed in a "pull down assay" in which S-tagged hrg was immobilized on S-protein resin (lanes 4-9) and HER3<sup>I-IV</sup> could be specifically eluted with 1 μM hrg and detergent (lane 6 and 8), but HER3<sup>I-IV</sup>E64A could not be eluted with PBS or 1μM hrg (lanes 5 and 7). A small amount of HER3<sup>I-IV</sup>E64A could be eluted with detergent (lane 9) which was probably due to some binding or non-specific interaction. Both HER3<sup>I-IV</sup> and HER3<sup>I-IV</sup>E64A showed little non-specific binding to S-protein resin without immobilized S-tagged hrg when eluted with 1μM hrg (lane 2-3). This shows that HER3<sup>I-IV</sup>E64A does not bind hrg.

Fig. 13. Space filling model of domains I, II, and III of the structure of IGF-1R showing the positions of the protected proteolysis site (red) and residues E64 (yellow) and V110 (green) in the HER3<sup>I-IV</sup> mapped to the IGF-1R structure (see, e.g. Garrett et al. (1998) *Nature* 394(6691), 395-9). The hormone binding footprint is in purple. E64 and V110 of HER3<sup>I-IV</sup> are in or near the binding footprint in domain L1 of IGF-1R.

Fig. 14. Residues 64 and 110 could form specific interactions with heregulin. A, Backbone representation of domain I of IGF-1R showing the position of E64 (yellow)

cpk) and V110 (green cpk) (H30 and L81 in IGF-1R) the residues critical for heregulin binding and the protected proteolysis site at position 50 in HER3<sup>I-IV</sup> (red cpk) (18 in IGF-1R) superimposed on the IGF-1R structure. E64 (H30 in IGF-1R) and V110 (L81 in IGF-1R) which are critical for heregulin binding are 12 Å from each other and E64 (H30 in IGF-1R) is 13 Å from residue 50 (position 18 in IGF-1R) the protected cleavage site. B, Backbone representation of the EGF-like domain of heregulin showing the position of H178 (yellow cpk) and V180 (green cpk). HER3 V110 and hrg V180 could form hydrophobic interactions. HER3 E64 and hrg H178 could form a salt bridge. These specific interactions could be disrupted by the mutations of E64A and V110A in HER3 as we observed in our biochemical experiments.

**Fig. 15.** The N-terminal residues H178 (yellow cpk) and V180 (green cpk) of hrg can form interactions with the residues E64 (yellow cpk) and V110 (green cpk) of HER3 and that the omega loop of hrg could block the protected proteolysis site (residue 50 red cpk) by sterically hindering interaction with trypsin.

**Fig. 16.** HER3<sup>FL</sup> (residues 20-1342) binds heregulin, but HER3<sup>FL E64A</sup> (residues 20-1342 with a mutation of E64A) and HER3<sup>II-CTD</sup> (Domain I in figure, residues 184-1342) do not. A, Specific binding of <sup>125</sup>I hrg to COS 7 cells transiently expressing HER3 mutants. The FL HER3 shows a high level of specific binding while the HER3<sup>FL E64A</sup> and HER3<sup>II-CTD</sup> show similar binding to cells expressing the vector only. B, Western blot analysis of transient expression of the HER3 mutants in COS7 cells. The WT (HER3<sup>FL</sup>), E64A, and Domain I all express similar levels of protein.

#### DETAILED DESCRIPTION OF THE INVENTION

Unless otherwise defined, all terms of art, notations and other scientific terms or terminology used herein are intended to have the meanings commonly understood by those of skill in the art to which this invention pertains. In some cases, terms with commonly understood meanings are defined herein for clarity and/or for ready reference, and the inclusion of such definitions herein should not necessarily be

construed to represent a substantial difference over what is generally understood in the art. Many of the techniques and procedures described or referenced herein are well understood and commonly employed using conventional methodology by those skilled in the art, such as, for example, the widely utilized molecular cloning methodologies 5 described in see Ausubel et al., Current Protocols in Molecular Biology, Wiley Interscience Publishers, (1995) and Sambrook et al., Molecular Cloning: A Laboratory Manual 2nd. edition (1989) Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y. As appropriate, procedures involving the use of commercially available kits and reagents are generally carried out in accordance with manufacturer defined protocols 10 and/or parameters unless otherwise noted.

Briefly, the disclosure provided herein identifies domains I-II of HER3 as the major binding region for heregulin and identifies residue positions in HER3 that, when mutated, produce heregulin binding polypeptides which have an altered affinity for this ligand. Prior to this discovery, the region of ligand binding in HER3 was unknown. 15 This domain localization is unexpected in view of observations of EGFR (in which the binding of its ligand occurs in domain III), a closely related receptor that has a sequence identity HER3 and which belongs in the same family of type 1 kinase receptors. See, e.g. Singer et al., JBC (2001), 276(47): 44266-44274. Various aspects and embodiments of the invention disclosed herein are provided in the description below.

20

### I. Abbreviations

Abbreviations used include: HER, human epidermal growth factor receptor; ECD, extracellular domain; EGF, epidermal growth factor; EGFR, epidermal growth factor receptor; hrg, 60-residue EGF-like domain of human heregulin 1; IGF-1R, insulin 25 growth factor-1 receptor; IR, insulin receptor; NA5-hrg, binding-deficient hrg mutant; PBS, phosphate-buffered saline; RTK, receptor tyrosine kinase; trx-hrg, thioredoxin-heregulin fusion;  $\beta$ -ME,  $\beta$ -mercaptoethanol; PAGE, polyacrylamide gel electrophoresis;

BSA, bovine serum albumin; MALDI, matrix-assisted laser desorption/ionization; SPR, surface plasmon resonance; MES, 4-morpholineethanesulfonic acid.

## II. Definitions

5        The terms "HER3", "HER3 polypeptide" or "HER3 receptor" when used herein encompass "native sequence HER3 polypeptides" and "HER3 variants" (which are further defined herein). "HER3" is a designation given to those polypeptides which are encoded by the nucleic acid molecules comprising the polynucleotide sequences shown in SEQ ID NO: 1 and variants or fragments thereof, nucleic acid molecules comprising 10 the sequence shown in the SEQ ID NO: 1. The HER3 polynucleotides and polypeptides of the invention may be isolated from a variety of sources, such as from human tissue types or from another source, or prepared by recombinant and/or synthetic methods. See, e.g. Plowman et al., PNAS (1990) 87: 4905-4909. As used herein, the term "polypeptide" means an amino acid polymer of at least 6 amino acids.

15       A "native sequence" HER3 polypeptide comprises a polypeptide having the same amino acid sequence as the corresponding HER3 polypeptide derived from nature. Such native sequence HER3 polypeptides can be isolated from nature or can be produced by recombinant and/or synthetic means. The term "native sequence HER3 polypeptide" specifically encompasses naturally-occurring truncated or secreted forms (e.g., an 20 extracellular domain sequence), naturally-occurring variant forms (e.g., alternatively spliced forms) and naturally-occurring allelic variants of the polypeptide. The HER3 polypeptides of the invention include the HER3 polypeptide comprising or consisting of the contiguous sequence of amino acid residues 1 to 1342 of SEQ ID NO: 2.

A HER3 "extracellular domain" or "ECD" refers to a form of the HER3 25 polypeptide which is essentially free of the transmembrane and cytoplasmic domains. Ordinarily, a HER3 polypeptide ECD will have less than about 1% of such transmembrane and/or cytoplasmic domains and preferably, will have less than about 0.5% of such domains. It will be understood that any transmembrane domain(s)

identified for the HER3 polypeptides of the present invention are identified pursuant to criteria routinely employed in the art for identifying that type of hydrophobic domain. The exact boundaries of a transmembrane domain may vary but most likely by no more than about 5 amino acids at either end of the domain as initially identified.

5 "HER3 variant" means a HER3 polypeptide having at least about 80% amino acid sequence identity with the amino acid sequence of a native sequence full length HER3 or HER3 ECD. Such HER3 variant polypeptides include, for instance, HER3 polypeptides wherein one or more amino acid residues are added, or deleted, at the N- and/or C-terminus, as well as within one or more internal domains, of the full-length 10 amino acid sequence. Fragments of the HER3 ECD are also contemplated. Ordinarily, a HER3 variant polypeptide will have at least about 80% amino acid sequence identity, more preferably at least about 81% amino acid sequence identity, more preferably at least about 82% amino acid sequence identity, more preferably at least about 83% amino acid sequence identity, more preferably at least about 84% amino acid sequence identity, more 15 preferably at least about 85% amino acid sequence identity, more preferably at least about 86% amino acid sequence identity, more preferably at least about 87% amino acid sequence identity, more preferably at least about 88% amino acid sequence identity, more preferably at least about 89% amino acid sequence identity, more preferably at least about 90% amino acid sequence identity, more preferably at least about 91% amino acid 20 sequence identity, more preferably at least about 92% amino acid sequence identity, more preferably at least about 93% amino acid sequence identity, more preferably at least about 94% amino acid sequence identity, more preferably at least about 95% amino acid sequence identity, more preferably at least about 96% amino acid sequence identity, more preferably at least about 97% amino acid sequence identity, more preferably at least about 98% amino acid sequence identity and yet more preferably at least about 99% 25 amino acid sequence identity with a HER3 polypeptide encoded by a nucleic acid molecule shown in Figure 10 or a specified fragment thereof. HER3 variant polypeptides do not encompass the native HER3 polypeptide sequence. Ordinarily,

HER3 variant polypeptides are at least about 10 amino acids in length, often at least about 20 amino acids in length, more often at least about 30 amino acids in length, more often at least about 40 amino acids in length, more often at least about 50 amino acids in length, more often at least about 60 amino acids in length, more often at least about 70  
5 amino acids in length, more often at least about 80 amino acids in length, more often at least about 90 amino acids in length, more often at least about 100 amino acids in length, more often at least about 150 amino acids in length, more often at least about 200 amino acids in length, more often at least about 250 amino acids in length, more often at least about 300 amino acids in length, or more.

10 The terms "heregulin" or "heregulin polypeptide" when used herein encompass "native sequence heregulin polypeptides" and "heregulin variants". "Heregulin" is a designation given to those polypeptides which are encoded by the nucleic acid molecules comprising the polynucleotide sequences shown in SEQ ID NO: 3 and variants thereof, nucleic acid molecules comprising the sequence shown in the SEQ ID NO: 3, and  
15 variants thereof as well as fragments of the above which have the biological activity of the native sequence heregulin. Variants of heregulin will preferably have at least 80%, more preferably, at least 90%, and even more preferably, at least 95% amino acid sequence identity with the native sequence heregulin polypeptide shown in SEQ ID NO:  
4. A "native sequence" heregulin polypeptide comprises a polypeptide having the same  
20 amino acid sequence as the corresponding heregulin polypeptide derived from nature. Such native sequence heregulin polypeptides can be isolated from nature or can be produced by recombinant and/or synthetic means.

The terms "HER2" or "HER2 polypeptide" when used herein encompass "native sequence HER2 polypeptides" and "HER2 variants". "HER2" is a designation  
25 given to those polypeptides which are encoded by the nucleic acid molecules comprising the polynucleotide sequences shown in SEQ ID NO: 5 and variants thereof, nucleic acid molecules comprising the sequence shown in the Figure SEQ ID NO: 5, and variants thereof as well as fragments of the above which have the biological activity of the native

sequence HER2. Variants of HER2 will preferably have at least 80%, more preferably, at least 90%, and even more preferably, at least 95% amino acid sequence identity with the native sequence HER2 polypeptide shown in SEQ ID NO: 6. A "native sequence" HER2 polypeptide comprises a polypeptide having the same amino acid sequence as the corresponding HER2 polypeptide derived from nature. Such native sequence HER2 polypeptides can be isolated from nature or can be produced by recombinant and/or synthetic means.

"Stringency" of hybridization reactions is readily determinable by one of ordinary skill in the art, and generally is an empirical calculation dependent upon probe length, washing temperature, and salt concentration. In general, longer probes require higher temperatures for proper annealing, while shorter probes need lower temperatures. Hybridization generally depends on the ability of denatured DNA to re-anneal when complementary strands are present in an environment below their melting temperature. The higher the degree of desired identity between the probe and hybridizable sequence, the higher the relative temperature which can be used. As a result, it follows that higher relative temperatures would tend to make the reaction conditions more stringent, while lower temperatures less so. For additional details and explanation of stringency of hybridization reactions, see Ausubel *et al*, Current Protocols in Molecular Biology, Wiley Interscience Publishers, (1995).

"Stringent conditions" or "high stringency conditions", as defined herein, are identified by those that: (1) employ low ionic strength and high temperature for washing, 0.015 M sodium chloride/0.0015 M sodium citrate/0.1% sodium dodecyl sulfate at 50°C; (2) employ during hybridization a denaturing agent, 50% (v/v) formamide with 0.1% bovine serum albumin/0.1% Ficoll/0.1% polyvinylpyrrolidone/50mM sodium phosphate buffer at pH 6.5 with 750 mM sodium chloride, 75 mM sodium citrate at 42°C; or (3) employ 50% formamide, 5 x SSC (0.75 M NaCl, 0.075 M sodium citrate), 50 mM sodium phosphate (pH 6.8), 0.1% sodium pyrophosphate, 5 x Denhardt's solution, sonicated salmon sperm DNA (50 µg/ml), 0.1% SDS, and 10% dextran sulfate at 42°C,

with washes at 42°C in 0.2 x SSC (sodium chloride/sodium citrate) and 50% formamide at 55°C, followed by a high-stringency wash consisting of 0.1 x SSC containing EDTA at 55°C.

"Moderately stringent conditions" are identified as described by Sambrook *et al.*,  
5 Molecular Cloning: A Laboratory Manual, New York: Cold Spring Harbor Press, 1989,  
and include the use of washing solution and hybridization conditions (e.g., temperature,  
ionic strength and %SDS) less stringent than those described above. An example of  
moderately stringent conditions is overnight incubation at 37°C in a solution comprising:  
20% formamide, 5 x SSC (150 mM NaCl, 15 mM trisodium citrate), 50 mM sodium  
10 phosphate (pH 7.6), 5 x Denhardt's solution, 10% dextran sulfate, and 20 mg/ml  
denatured sheared salmon sperm DNA, followed by washing the filters in 1 x SSC at  
about 37-50°C. The skilled artisan will recognize how to adjust the temperature, ionic  
strength, etc. as necessary to accommodate factors such as probe length and the like.

Nucleic acid is "operably linked" when it is placed into a functional relationship  
15 with another nucleic acid sequence. For example, DNA for a presequence or secretory  
leader is operably linked to DNA for a polypeptide if it is expressed as a preprotein that  
participates in the secretion of the polypeptide; a promoter or enhancer is operably  
linked to a coding sequence if it affects the transcription of the sequence; or a ribosome  
binding site is operably linked to a coding sequence if it is positioned so as to facilitate  
20 translation. Generally, "operably linked" means that the DNA sequences being linked  
are contiguous, and, in the case of a secretory leader, contiguous and in reading phase.  
However, enhancers do not have to be contiguous. Linking is accomplished by ligation  
at convenient restriction sites. If such sites do not exist, the synthetic oligonucleotide  
adaptors or linkers are used in accordance with conventional practice.

25 The terms "amino acid" and "amino acids" refer to all naturally occurring L-  
alpha-amino acids. This definition is meant to include norleucine, ornithine, and  
homocysteine. The amino acids are identified by either the single-letter or three-letter  
designations:

	Asp D aspartic acid	Ile I isoleucine
	Thr T threonine	Leu L leucine
	Ser S serine	Tyr Y tyrosine
	Glu E glutamic acid	Phe F phenylalanine
5	Pro P proline	His H histidine
	Gly G glycine	Lys K lysine
	Ala A alanine	Arg R arginine
	Cys C cysteine	Trp W tryptophan
	Val V valine	Gln Q glutamine
10	Met M methionine	Asn N asparagine

In the Sequence Listing and Figures, certain other single-letter or three-letter designations may be employed to refer to and identify two or more amino acids or nucleotides at a given position in the sequence.

- "Percent (%) amino acid sequence identity" with respect to the ligand or receptor polypeptide sequences identified herein is defined as the percentage of amino acid residues in a candidate sequence that are identical with the amino acid residues in such a ligand or receptor sequence identified herein, after aligning the sequences and introducing gaps, if necessary, to achieve the maximum percent sequence identity, and not considering any conservative substitutions as part of the sequence identity.
- Alignment for purposes of determining percent amino acid sequence identity can be achieved in various ways that are within the skill in the art, for instance, using publicly available computer software such as BLAST, BLAST-2, ALIGN, ALIGN-2 or Megalign (DNASTAR) software. Those skilled in the art can determine appropriate parameters for measuring alignment, including any algorithms needed to achieve maximal alignment over the full-length of the sequences being compared.

The term "antagonist" is used in the broadest sense, and includes any molecule that partially or fully blocks, inhibits, or neutralizes one or more biological activities of heregulin polypeptide, HER3 polypeptide, or both heregulin and HER3, *in vitro*, *in situ*, or

in vivo. Examples of such biological activities of heregulin and HER3 polypeptides include binding of heregulin to HER3 and activation of the mitogenic response that is observed when heregulin interacts with HER3, as reported in the literature (see, e.g. Lee et al., (2001) Cancer Research 61, 4467-4473; Aguilar et al., (1999) Oncogene 18, 6050-6052; Marte et al., Oncogene 1995 Jan 5;10(1):167-75; and Neve et al., Oncogene 2000 Mar 23;19(13):1647-56). An antagonist may function in a direct or indirect manner. For instance, the antagonist may function to partially or fully block, inhibit or neutralize one or more biological activities of heregulin polypeptide, HER3 polypeptide, or both heregulin and HER3, *in vitro*, *in situ*, or *in vivo* as a result of their direct interaction. The antagonist may also function indirectly to partially or fully block, inhibit or neutralize one or more biological activities of heregulin polypeptide, HER3 polypeptide, or both heregulin and HER3, *in vitro*, *in situ*, or *in vivo* as a result of, e.g., blocking or inhibiting another effector molecule. The antagonist molecule may comprise a "dual" antagonist activity wherein the molecule is capable of partially or fully blocking, inhibiting or neutralizing a biological activity of both heregulin and HER3.

The term "agonist" is used in the broadest sense, and includes any molecule that partially or fully enhances, stimulates or activates one or more biological activities of heregulin polypeptide, HER3 polypeptide, or both heregulin and HER3, *in vitro*, *in situ*, or *in vivo*. Examples of such biological activities of heregulin and HER3 may include activation of downstream signal transduction (see, e.g. Aguilar et al., (1999) Oncogene 18, 6050-6052; Marte et al., Oncogene 1995 Jan 5;10(1):167-75; and Neve et al., Oncogene 2000 Mar 23;19(13):1647-56). An agonist may function in a direct or indirect manner. For instance, the agonist may function to partially or fully enhance, stimulate or activate one or more biological activities of heregulin polypeptide, HER3 polypeptide, or both heregulin and HER3, *in vitro*, *in situ*, or *in vivo* as a result of its direct binding to heregulin or HER3, which causes receptor activation or signal transduction. The agonist may also function indirectly to partially or fully enhance, stimulate or activate one or more biological activities of heregulin polypeptide, HER3 polypeptide, or both heregulin

and HER3, *in vitro*, *in situ*, or *in vivo* as a result of, e.g., stimulating another effector molecule which then causes heregulin or HER3 receptor activation or signal transduction. It is contemplated that an agonist may act as an enhancer molecule which functions indirectly to enhance or increase heregulin or HER3 activation or activity. For 5 instance, the agonist may enhance activity of endogenous heregulin or HER3 in a mammal. This could be accomplished, for example, by pre-complexing heregulin or HER3 or by stabilizing complexes of the respective ligand with the heregulin or HER3 receptor (such as stabilizing native complex formed between heregulin and HER3).

The term "heregulin antagonist" or "HER3 antagonist" refers to any molecule that 10 partially or fully blocks, inhibits, or neutralizes a biological activity of heregulin or HER3, respectively, or both heregulin and HER3, and include, but are not limited to, soluble forms of HER3 receptor or such as an extracellular domain sequence of HER3, HER3 receptor fusion proteins, covalently modified forms of HER3 receptor, covalently modified forms of HER3 receptor, HER3 variants, heregulin variants, HER3 receptor antibodies 15 and heregulin antibodies. To determine whether a heregulin antagonist molecule partially or fully blocks, inhibits or neutralizes a biological activity of heregulin or HER3, assays may be conducted to assess the effect(s) of the antagonist molecule on, for example, binding of heregulin to HER3. Such assays may be conducted in known *in vitro* or *in vivo* assay formats, for instance, in cells expressing HER3. Preferably, the heregulin antagonist 20 employed in the methods described herein will be capable of blocking or neutralizing at least one type of heregulin activity, which may optionally be determined in assays such as described herein. To determine whether an HER3 antagonist molecule partially or fully blocks, inhibits or neutralizes a biological activity of heregulin or HER3, assays may be conducted to assess the effect(s) of the antagonist molecule on, for example, binding of 25 heregulin to HER3. Such assays may be conducted in known *in vitro* or *in vivo* formats, for instance, using cells transfected with HER3. Preferably, the HER3 antagonist employed in the methods described herein will be capable of blocking or neutralizing at least one type of HER3 activity. Optionally, a heregulin antagonist or HER3 antagonist will be capable of

reducing or inhibiting binding of heregulin to HER3 by at least 50%, preferably, by at least 90%, more preferably by at least 99%, and most preferably, by 100%, as compared to a negative control molecule, in a binding assay. In one embodiment, the heregulin antagonist or HER3 antagonist will comprise antibodies which will competitively inhibit the binding 5 of heregulin to HER3. Methods for determining antibody specificity and affinity by competitive inhibition are known in the art (see, e.g., Harlow et al., Antibodies: A Laboratory Manual, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY (1998); Colligan et al., Current Protocols in Immunology, Green Publishing Assoc., NY (1992; 1993); Muller, Meth. Enzym., 92:589-601 (1983)).

10       The term "heregulin agonist" or "HER3 agonist" refers to any molecule that partially or fully enhances, stimulates or activates a biological activity of heregulin or HER3, respectively, or both heregulin and HER3, and include, but are not limited to HER3 variant polypeptides and heregulin binding domain specific anti-HER3 receptor antibodies. To determine whether a heregulin agonist molecule partially or fully enhances, stimulates, or 15 activates a biological activity of HER3, assays may be conducted to assess the effect(s) of the agonist molecule on, for example, HER3-expressing or HER3-transfected cells. Such assays may be conducted in known *in vitro* or *in vivo* assay formats. Preferably, the heregulin agonist employed in the methods described herein will be capable of enhancing or activating at least one type of heregulin activity, which may optionally be determined in 20 assays such as described herein.

25       The term "antibody" is used in the broadest sense and specifically covers, for example, single monoclonal antibodies against heregulin and HER3 (e.g. those targeting the heregulin binding domain in HER3), antibody compositions with polyepitopic specificity, single chain antibodies, and fragments of antibodies. "Antibody" as used herein includes intact immunoglobulin or antibody molecules, polyclonal antibodies, multispecific antibodies (i.e., bispecific antibodies formed from at least two intact antibodies) and immunoglobulin fragments (such as Fab, F(ab')<sub>2</sub>, or Fv), so long as they exhibit any of the desired agonistic or antagonistic properties described herein.

Antibodies are typically proteins or polypeptides which exhibit binding specificity to a specific antigen. Native antibodies are usually heterotetrameric glycoproteins, composed of two identical light (L) chains and two identical heavy (H) chains. Typically, each light chain is linked to a heavy chain by one covalent disulfide bond, while the number 5 of disulfide linkages varies between the heavy chains of different immunoglobulin isotypes. Each heavy and light chain also has regularly spaced intrachain disulfide bridges. Each heavy chain has at one end a variable domain ( $V_H$ ) followed by a number of constant domains. Each light chain has a variable domain at one end ( $V_L$ ) and a constant domain at its other end; the constant domain of the light chain is aligned with the first constant 10 domain of the heavy chain, and the light chain variable domain is aligned with the variable domain of the heavy chain. Particular amino acid residues are believed to form an interface between the light and heavy chain variable domains (Chothia et al., *J. Mol. Biol.*, **186**:651-663 (1985); Novotny and Haber, *Proc. Natl. Acad. Sci. USA*, **82**:4592-4596 (1985)). The light chains of antibodies from any vertebrate species can be assigned to one of two clearly 15 distinct types, called kappa and lambda, based on the amino acid sequences of their constant domains. Depending on the amino acid sequence of the constant domain of their heavy chains, immunoglobulins can be assigned to different classes. There are five major classes of immunoglobulins: IgA, IgD, IgE, IgG and IgM, and several of these may be further divided into subclasses (isotypes), e.g., IgG-1, IgG-2, IgG-3, and IgG-4; IgA-1 and 20 IgA-2. The heavy chain constant domains that correspond to the different classes of immunoglobulins are called alpha, delta, epsilon, gamma, and mu, respectively.

"Antibody fragments" comprise a portion of an intact antibody, generally the antigen binding or variable region of the intact antibody. Examples of antibody fragments include Fab, Fab', F(ab')2, and Fv fragments, diabodies, single chain antibody molecules, 25 and multispecific antibodies formed from antibody fragments.

The term "variable" is used herein to describe certain portions of the variable domains which differ in sequence among antibodies and are used in the binding and specificity of each particular antibody for its particular antigen. However, the variability is

not usually evenly distributed through the variable domains of antibodies. It is typically concentrated in three segments called complementarity determining regions (CDRs) or hypervariable regions both in the light chain and the heavy chain variable domains. The more highly conserved portions of the variable domains are called the framework (FR).

5     The variable domains of native heavy and light chains each comprise four FR regions, largely adopting a  $\beta$ -sheet configuration, connected by three CDRs, which form loops connecting, and in some cases forming part of, the  $\beta$ -sheet structure. The CDRs in each chain are held together in close proximity by the FR regions and, with the CDRs from the other chain, contribute to the formation of the antigen binding site of antibodies (see

10    Kabat, E.A. et al., Sequences of Proteins of Immunological Interest, National Institutes of Health, Bethesda, MD (1987)). The constant domains are not involved directly in binding an antibody to an antigen, but exhibit various effector functions, such as participation of the antibody in antibody-dependent cellular toxicity.

The term "monoclonal antibody" as used herein refers to an antibody obtained  
15    from a population of substantially homogeneous antibodies, i.e., the individual antibodies comprising the population are identical except for possible naturally-occurring mutations that may be present in minor amounts. Monoclonal antibodies are highly specific, being directed against a single antigenic site. Furthermore, in contrast to conventional (polyclonal) antibody preparations which typically include different antibodies directed  
20    against different determinants (epitopes), each monoclonal antibody is directed against a single determinant on the antigen.

The monoclonal antibodies herein include chimeric, hybrid and recombinant antibodies produced by splicing a variable (including hypervariable) domain of the antibody of interest with a constant domain (e.g. "humanized" antibodies), or a light chain with a  
25    heavy chain, or a chain from one species with a chain from another species, or fusions with heterologous proteins, regardless of species of origin or immunoglobulin class or subclass designation, as well as antibody fragments (e.g., Fab, F(ab)<sub>2</sub>, and Fv), so long as they exhibit the desired biological activity or properties. See, e.g. U.S. Pat. No. 4,816,567 and Mage et

al., in Monoclonal Antibody Production Techniques and Applications, pp.79-97 (Marcel Dekker, Inc.: New York, 1987).

The term "immunospecific" as used in "immunospecific binding of antibodies" for example, refers to the antigen specific binding interaction that occurs between the 5 antigen-combining site of an antibody and the specific antigen recognized by that antibody.

"Isolated," when used to describe the various proteins disclosed herein, means protein that has been identified and separated and/or recovered from a component of its natural environment. Contaminant components of its natural environment are materials 10 that would typically interfere with diagnostic or therapeutic uses for the protein, and may include enzymes, hormones, and other proteinaceous or non-proteinaceous solutes. In preferred embodiments, the protein will be purified (1) to a degree sufficient to obtain at least 15 residues of N-terminal or internal amino acid sequence by use of a spinning cup sequenator, or (2) to homogeneity by SDS-PAGE under non-reducing or reducing 15 conditions using Coomassie blue or, preferably, silver stain. Isolated protein includes protein *in situ* within recombinant cells, since at least one component of the protein natural environment will not be present. Ordinarily, however, isolated protein will be prepared by at least one purification step.

"Treatment" or "therapy" refer to both therapeutic treatment and prophylactic or 20 preventative measures.

"Mammal" for purposes of treatment or therapy refers to any animal classified as a mammal, including humans, domestic and farm animals, and zoo, sports, or pet animals, such as dogs, horses, cats, cows, *etc.* Preferably, the mammal is human.

"HER3-related pathological condition" and "hereregulin-related pathological 25 condition" refer to pathologies or conditions associated with abnormal levels of expression or activity of HER3 or hereregulin, respectively, in excess of, or less than, levels of expression or activity in normal healthy mammals, where such excess or diminished levels occur in a systemic, localized, or particular tissue or cell type or location in the

body. HER3 -related pathological conditions and heregulin-related pathological conditions include syndromes characterized by disregulated cell growth such as cancer.

The terms "cancer", "cancerous", and "malignant" refer to or describe the physiological condition in mammals that is typically characterized by unregulated cell growth. Examples of cancer include but are not limited to, carcinoma including adenocarcinoma, lymphoma, blastoma, melanoma, sarcoma, and leukemia. More particular examples of such cancers include squamous cell cancer, small-cell lung cancer, non-small cell lung cancer, gastrointestinal cancer, Hodgkin's and non-Hodgkin's lymphoma, pancreatic cancer, glioblastoma, cervical cancer, ovarian cancer, liver cancer such as hepatic carcinoma and hepatoma, bladder cancer, breast cancer, colon cancer, colorectal cancer, endometrial carcinoma, myeloma (such as multiple myeloma), salivary gland carcinoma, kidney cancer such as renal cell carcinoma and Wilms' tumors, basal cell carcinoma, melanoma, prostate cancer, vulval cancer, thyroid cancer, testicular cancer, esophageal cancer, and various types of head and neck cancer. Optionally, the cancer will express, or have associated with the cancer cell, heregulin or HER3. By way of example, breast and ovarian cancers are among the cancers reported in the literature to express HER3.

## II. Methods and Materials

The disclosure provided herein teaches that the EGF-like domain of heregulin (hrg) binds to domains I and II of HER3, in contrast to the EGF receptor, for which prior studies have shown that a construct consisting of domains III and portions of domain IV binds EGF. As illustrated in Example 2, a hrg binding site is identified by limited proteolysis of the recombinant extracellular domains of HER3 (HER3-ECD<sup>I-IV</sup>) in both the presence and absence of hrg. In the absence of hrg, HER3-ECD<sup>I-IV</sup> is cleaved after position Tyr<sup>50</sup>, near the beginning of domain I. Binding of hrg to HER3-ECD<sup>I-IV</sup> fully protects position Tyr<sup>50</sup> from proteolysis. To confirm that domain I contains a hrg binding site, a HER3 polypeptide comprising only domains I and II (and

not domains III and IV) of HER3 (HER3-ECD<sup>I-II</sup>) was generated. This HER3-ECD<sup>I-II</sup> polypeptide was then shown to bind hrg with 68 nM affinity. These data provide evidence that domains I and II of HER3-ECD<sup>I-IV</sup> act as a functional unit in folding and binding of hrg.

5       The biochemical findings reinforce the structural hypothesis that HER3-ECD<sup>I-IV</sup> is similar to the insulin-like growth factor-1 receptor (IGF-1R), as follows: 1) The protected cleavage site in HER3-ECD<sup>I-IV</sup> corresponds to a binding footprint in domain I of IGF-1R; 2) HER3-ECD<sup>I-II</sup> binds hrg with a 68 nM dissociation constant, supporting the hypothesis that domain I is involved in ligand binding; and 3) the large accessible  
10 surface area (1749 Å) of domain L1 of IGF-1R that is buried by domain S1, as well as the presence of conserved contacts in this interface of type 1 RTKs, provide evidences that domains L1 and S1 of IGF-1R function as a unit as observed for HER3-ECD<sup>I-II</sup>. The results are consistent with a model wherein HER3 has a structure similar to IGF-1R and binds ligand at a site in corresponding domains.

15       In this context, the invention provides methods and materials for identifying molecules which bind to the heregulin binding site on HER3 as well as methods and materials for modulating heregulin and/or HER3 activity in mammalian cells. Typical methods for modulating heregulin and/or HER3 activity in mammalian cells comprise exposing the cells to a desired amount of antagonist or agonist that affects heregulin  
20 interaction with HER3. Preferably, the amount of antagonist or agonist employed will be an amount effective to affect the binding and/or activity of the respective ligand or respective receptor to achieve a therapeutic effect. This can be accomplished *in vivo* or *ex vivo* in accordance, for instance, with the methods described below and in the Examples. An exemplary condition or disorders to be treated with such antagonists or antagonists is  
25 a cancer whose malignant phenotype is associated with aberrant HER3 or heregulin expression.

Diagnostic methods are also provided herein. For instance, the antagonists or agonists may be employed to detect the respective binding partner (heregulin and HER3)

or in mammals known to be or suspected of having a heregulin-related pathological condition or HER3-related pathological condition. The antagonist or agonist molecule may be used, e.g., in immunoassays to detect or quantitate heregulin or HER3 in a sample. A sample, such as cells obtained from a mammal, can be incubated in the presence of a labeled antagonist or agonist molecule, and detection of the labeled antagonist or agonist bound in the sample can be performed. Such assays, including various clinical assay procedures, are known in the art.

The antagonists and agonists which can be employed in the methods include, but are not limited to, soluble forms of HER3 receptor, HER3 receptor immunoadhesins, fusion proteins comprising HER3, covalently modified forms of heregulin and HER3, HER3 receptor variants anti-HER3 receptor antibodies that target the heregulin binding domain, and anti-heregulin antibodies that target the HER3 binding domain. Various techniques that can be employed for making the antagonists and agonists are described herein.

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#### A. HER3 POLYPEPTIDES

The disclosure provided herein includes HER3 polypeptides such as the HER3-ECD<sup>I-II</sup> polypeptide discussed above as well as HER3 substitution variants such as those having an amino acid substitution at residue E64 and/or V110. The description below provides typical schemes for the production of HER3 polypeptides including HER3 polypeptide variants, by culturing cells transformed or transfected with a vector containing HER3 polypeptide encoding nucleic acid. It is, of course, contemplated that alternative methods, which are well known in the art, may be employed to prepare HER3 polypeptides. For instance, the HER3 polypeptide sequence, or portions thereof, may be produced by direct peptide synthesis using solid-phase techniques (see, e.g., Stewart et al, Solid-Phase Peptide Synthesis, W.H. Freeman Co., San Francisco, CA (1969); Merrifield, J. Am. Chem. Soc., 85:2149-2154 (1963)). *In vitro* protein synthesis may be performed using manual techniques or by automation. Automated synthesis may be accomplished,

for instance, using an Applied Biosystems Peptide Synthesizer (Foster City, CA) using manufacturer's instructions. Various portions of HER3 polypeptides may be chemically synthesized separately and combined using chemical or enzymatic methods to produce a full-length HER3 polypeptide.

5 This disclosure identifies amino acids in discontinuous segments in domain I of HER3 which appear to be critical for heregulin binding (see, e.g. Table 3). For example, a mutation at residue 64 appears to decrease all binding affinity in both recombinant HER3 I-IV E64A and HER3 I-II E64A; while a mutation of V110A in and HER3 I-II causes a 4 fold increase in heregulin binding affinity. Residues 64 and 110 are in domain I of  
10 HER3 and map to residues H30 and L81 in the hormone binding footprint in the IGF-1R. Residues H30 and L81 lie on different strands in IGF-1R, but are within 12A of each other. HER3 V110 and hrg V180 could form hydrophobic interactions, and HER3 E64 and hrg H178 could form a salt bridge with heregulin. These specific interactions could be disrupted by the mutations of E64A and V110A in HER3 as we observed in  
15 our biochemical experiments.

A preferred embodiment of the invention is a HER3 variant polypeptide comprising an amino acid sequence which differs from the native sequence HER3 polypeptide sequence of SEQ ID NO: 2 and has one or more of amino acid substitutions at the following residue position(s) in SEQ ID NO: 2: E43; N44; K51; E64;  
20 V66; and V110. Typically, the variant binds heregulin with an affinity different from a HER2 polypeptide that has no amino acid substitution at the enumerated position(s). Another preferred embodiment of the invention is a HER3 variant polypeptide comprising an amino acid sequence which differs from the native sequence HER3 polypeptide sequence of SEQ ID NO: 2 and has one or more of amino acid substitutions at the residue positions identified in Table 3. Another embodiment of the  
25 invention is a non-naturally occurring HER3 variant polypeptide consisting essentially of amino acid 20 to amino acid 329 of HER3 polypeptide sequence of SEQ ID NO: 2; wherein the HER3 variant polypeptide specifically binds to the heregulin polypeptide of

- SEQ ID NO: 4 and exhibits an impaired ability to interact with the HER2 polypeptide of SEQ ID NO: 6. Related embodiments of this invention include an isolated nucleic acid comprising a nucleotide sequence encoding these HER3 variants, vectors comprising nucleic acids encoding these variants and host cells (e.g. *E. coli*) containing these vectors.
- 5 Another related embodiment of the invention is a method of making HER3 variant polypeptide by providing a host cell comprising a vector encoding a HER3 variant, providing culture media, culturing the host cell in the culture media under conditions sufficient to express the HER3 variant polypeptide, recovering the HER3 variant polypeptide from the host cell or culture media, and then purifying the HER3 variant polypeptide. Optionally the HER3 variant polypeptide is conjugated or linked to one or more polyol groups.
- 10

The HER3 variants disclosed herein have a number of uses, and can, for example, be used to elucidate the signalling mechanisms of the heregulin/HER2/HER3 complex, a complex which, as noted above, is implicated in the malignant phenotype of certain cancers such as breast and ovarian cancers. Specifically, as noted herein, the molecules of the heregulin/HER2/HER3 complex contain different domains having different functional properties including an ability to associate in HER3 homo-oligomers as well as HER2/HER3 hetero-oligomers. The HER2/HER3 hetero-oligomers associated with heregulin produce a functional signalling complex that is associated with the biological activity of the molecules. Consequently, the variants disclosed herein which are designed to target a HER3 domain that binds to heregulin and to, for example, bind with a greater or lesser affinity to heregulin, can be used to assess interaction between HER2 and HER3 as well as the interaction between heregulin and HER2 and HER3. As is known in the art, the elucidation of structural domains associated with biological activities and the signalling mechanisms of molecules that are associated with pathologies such as cancer is a crucial step in the identification of novel diagnostic and therapeutic methods and materials (e.g. heregulin agonists and antagonists) which can be used to treat such pathologies.

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In this regard, the disclosure provides evidence that a specific subset of amino acid residues in the HER3 polypeptide are likely to be involved in the interaction with heregulin (and consequently can be mutated in studies designed to characterize the Her2, HER3 and heregulin interactions). The data providing this evidence includes a number 5 of independent and complementary observations including: (1) sequence comparison with residues in the structurally homologous IGF-1R molecule that are known to be associated with ligand binding (see, e.g. Figure 8); (2) crystallographic studies to map a heregulin binding footprint in HER3 (see, e.g. Figure 9); and (3) the further limitation of this subset to those residues that are most likely exposed to the solvent environment and 10 therefore likely to interact with heregulin.

For example, certain residues were chosen for mutation based on their proximity to the proteolysis site in domain I of HER3 (see, e.g. Singer et al. (2001) *J Biol Chem* 276(47), 44266-74), conservation to equivalent residues which are critical for ligand binding in the IR (see, e.g. Williams et al. (1995) *J Biol Chem* 270(7), 3012-6) and in the 15 hormone binding footprint in the IGF-1R (see, e.g. Garrett et al. (1998) *Nature* 394(6691), 395-9) based on a multiple sequence alignment performed between HER3, IR and IGF-1R. As noted above, this subset of HER3 residues includes those shown in Tables 2 and 3. The specific results obtained with the examination of members of this subset of HER3 residues (e.g. V110 and E64) provides further evidence that the 20 members of this specific subset of amino acid residues in the HER3 polypeptide are involved in the interaction with heregulin.

The HER3 variants disclosed herein include those where the wild type amino acid residue at one of the enumerated positions is substituted with any one of the other 19 naturally occurring amino acids. As is known in the art, there is a reasonable 25 expectation that a substitution at a wild type residue identified as likely to control affinity (for example residue 110) will effect the affinity of the HER3 variant for heregulin. Specifically, the pertinent art teaches that in situations where an experimental analysis has established that the properties of a specific residue at a particular position within the

polypeptide chain are likely involved in maintaining some aspect of a protein's functional integrity, an alteration in the size, shape, charge, hydrogen-bonding capacity or chemical reactivity of the amino acid side chain at one of these "active" amino acid positions is likely to affect the function of the protein in some way. For this reason, the skilled artisan would reasonably expect a perturbation in a HER3 residue likely to control the affinity of HER3 for heregulin to effect the affinity of HER3 for heregulin.

The invention further provides recombinant DNA or RNA molecules containing a HER3 polynucleotide, fragment, variant thereof, including but not limited to phages, plasmids, phagemids, cosmids, YACs, BACs, as well as various viral and non-viral vectors well known in the art, and cells transformed or transfected with such recombinant DNA or RNA molecules. Methods for generating such molecules are well known (see, for example, Sambrook et al, 1989, *supra*). The invention further provides a host-vector system comprising a recombinant DNA molecule containing a HER3 polynucleotide, fragment, variant thereof within a suitable prokaryotic or eukaryotic host cell. Examples of suitable eukaryotic host cells include a yeast cell, a plant cell, or an animal cell, such as a mammalian cell or an insect cell (e.g., a baculovirus-infectible cell such as an Sf9 or HighFive cell). Examples of suitable mammalian cells include a number of mammalian cells routinely used for the expression of recombinant proteins (e.g., COS, CHO, 293, 293T cells). More particularly, a polynucleotide comprising the coding sequence of HER3 or a fragment, variant thereof can be used to generate HER3 proteins or fragments thereof using any number of host-vector systems routinely used and widely known in the art.

A wide range of host-vector systems suitable for the expression of HER3 proteins or fragments thereof are available, see for example, Sambrook et al., 1989, *supra*; Current Protocols in Molecular Biology, 1995, *supra*). Preferred vectors for mammalian expression include but are not limited to pcDNA 3.1 myc-His-tag (Invitrogen) and the retroviral vector pSRαtkneo (Muller et al., 1991, MCB 11:1785). The host-vector systems of the invention are useful for the production of a HER3 protein or fragment

thereof. Such host-vector systems can be employed to study the functional properties of HER3 and HER3 mutations or analogs.

Recombinant human HER3 protein can be produced by mammalian cells transfected with a construct encoding a HER3 nucleotide. For example, 293T cells can  
5 be transfected with an expression plasmid encoding HER3 or fragment, variant thereof, the HER3 or related protein is expressed in the 293T cells, and the recombinant HER3 protein is isolated using standard purification methods (e.g., affinity purification using anti-HER3 antibodies). In another embodiment, a HER3 coding sequence is subcloned into the retroviral vector pSRαMSVtkneo and used to infect various mammalian cell  
10 lines, such as NIH 3T3, COS, CHO and MCF-7 in order to establish HER3 expressing cell lines. Various other expression systems well known in the art can also be employed. Expression constructs encoding a leader peptide joined in frame to the HER3 coding sequence can be used for the generation of a secreted form of recombinant HER3 protein.

15 As discussed herein, redundancy in the genetic code permits variation in HER3 gene sequences. In particular, it is known in the art that specific host species often have specific codon preferences, and thus one can adapt the disclosed sequence as preferred for a desired host. For example, preferred analog codon sequences typically have rare codons (i.e., codons having a usage frequency of less than about 20% in known  
20 sequences of the desired host) replaced with higher frequency codons.

Additional sequence modifications are known to enhance protein expression in a cellular host. These include elimination of sequences encoding spurious polyadenylation signals, exon/intron splice site signals, transposon-like repeats, and/or other such well-characterized sequences that are deleterious to gene expression. The GC content of the  
25 sequence is adjusted to levels average for a given cellular host, as calculated by reference to known genes expressed in the host cell. Where possible, the sequence is modified to avoid predicted hairpin secondary mRNA structures. Other useful modifications include

the addition of a translational initiation consensus sequence at the start of the open reading frame, as described in Kozak, *Mol. Cell Biol.*, 9:5073-5080 (1989).

Embodiments of the invention disclosed herein include a wide variety of art-accepted variants of HER3 proteins such as polypeptides having amino acid insertions, 5 deletions and substitutions. HER3 variants can be made using methods known in the art such as site-directed mutagenesis, alanine scanning, and PCR mutagenesis. Site-directed mutagenesis (Carter et al., *Nucl. Acids Res.*, 13:4331 (1986); Zoller et al., *Nucl. Acids Res.*, 10:6487 (1987)), cassette mutagenesis (Wells et al., *Gene*, 34:315 (1985)), restriction selection mutagenesis (Wells et al., *Philos. Trans. R. Soc. London Ser A*, 317:415 (1986)) or 10 other known techniques can be performed on the cloned DNA to produce the HER3 variant DNA.

Scanning amino acid analysis can also be employed to identify one or more amino acids along a contiguous sequence that is involved in a specific biological activity such as a protein-protein interaction. Among the preferred scanning amino acids are 15 relatively small, neutral amino acids. Such amino acids include alanine, glycine, serine, and cysteine. Alanine is typically a preferred scanning amino acid among this group because it eliminates the side-chain beyond the beta-carbon and is less likely to alter the main-chain conformation of the variant. Alanine is also typically preferred because it is the most common amino acid. Further, it is frequently found in both buried and 20 exposed positions (Creighton, *The Proteins*, (W.H. Freeman & Co., N.Y.); Chothia, *J. Mol. Biol.*, 150:1 (1976)). If alanine substitution does not yield adequate amounts of variant, an isosteric amino acid can be used.

HER3 proteins can be generated using standard peptide synthesis technology or using chemical cleavage methods well known in the art. Alternatively, recombinant 25 methods can be used to generate nucleic acid molecules that encode a HER3 protein. In one embodiment, nucleic acid molecules provide a means to generate defined fragments of the HER3 protein (or variants thereof).

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Modifications of HER3 proteins such as covalent modifications are included within the scope of this invention. One type of covalent modification includes reacting targeted amino acid residues of a HER3 polypeptide with an organic derivatizing agent that is capable of reacting with selected side chains or the N- or C-terminal residues of 5 the HER3. Another type of covalent modification of the HER3 polypeptide included within the scope of this invention comprises altering the native glycosylation pattern of a protein of the invention. Another type of covalent modification of HER3 comprises linking the HER3 polypeptide to one of a variety of nonproteinaceous polymers, e.g., polyethylene glycol (PEG), polypropylene glycol, or polyoxyalkylenes, in the manner set 10 forth in U.S. Patent Nos. 4,640,835; 4,496,689; 4,301,144; 4,670,417; 4,791,192 or 4,179,337.

#### B. ANTIBODIES

It is contemplated that anti-HER3 receptor antibodies that specifically target the 15 heregulin binding domain in HER3 may also be employed in the presently disclosed methods. Examples of such molecules include neutralizing or blocking antibodies which can preferably inhibit binding of heregulin to HER3 receptors. The anti-HER3 antibodies may be monoclonal antibodies.

Monoclonal antibodies may be prepared using hybridoma methods, such as those 20 described by Kohler and Milstein, *Nature*, 256:495 (1975). In a hybridoma method, a mouse, hamster, or other appropriate host animal, is typically immunized with an immunizing agent to elicit lymphocytes that produce or are capable of producing antibodies that will specifically bind to the immunizing agent. Alternatively, the lymphocytes may be immunized *in vitro*.

The immunizing agent will typically include a HER3 polypeptide or a fusion 25 protein thereof, such as a HER3 ECD-IgG fusion protein. The immunizing agent may alternatively comprise a fragment or portion of HER3 having one or more amino acids that participate in the binding of HER3 to heregulin. In a preferred embodiment, the

throughput screening of chemical libraries, making them particularly suitable for identifying small molecule drug candidates.

The assays can be performed in a variety of formats, including protein-protein binding assays, biochemical screening assays, immunoassays, and cell-based assays, which 5 are well characterized in the art. Assays for, for instance, antagonists are common in that they call for contacting the drug candidate with a ligand or receptor polypeptide identified herein under conditions and for a time sufficient to allow these two components to interact.

In binding assays, the interaction is binding and the complex formed can be 10 isolated or detected in the reaction mixture. In a particular embodiment, a preferred receptor polypeptide identified herein (e.g. HER3 polypeptide having only domains I and II) or the drug candidate is immobilized on a solid phase, e.g., on a microtiter plate, by covalent or non-covalent attachments. Non-covalent attachment generally is accomplished by coating the solid surface with a solution of the ligand or receptor 15 polypeptide and drying. Alternatively, an immobilized antibody, e.g., a monoclonal antibody, specific for the ligand or receptor polypeptide to be immobilized can be used to anchor it to a solid surface. The assay is performed by adding the non-immobilized component, which may be labeled by a detectable label, to the immobilized component, e.g., the coated surface containing the anchored component. When the reaction is 20 complete, the non-reacted components are removed, e.g., by washing, and complexes anchored on the solid surface are detected. When the originally non-immobilized component carries a detectable label, the detection of label immobilized on the surface indicates that complexing occurred. Where the originally non-immobilized component does not carry a label, complexing can be detected, for example, by using a labeled 25 antibody specifically binding the immobilized complex.

If the candidate compound interacts with but does not bind to a particular ligand or receptor polypeptide identified herein, its interaction with that polypeptide can be assayed by methods well known for detecting protein-protein interactions. Such assays

immunizing agent comprises an extracellular domain sequence of HER3 fused to an IgG sequence.

Generally, either peripheral blood lymphocytes ("PBLs") are used if cells of human origin are desired, or spleen cells or lymph node cells are used if non-human mammalian sources are desired. The lymphocytes are then fused with an immortalized cell line using a suitable fusing agent, such as polyethylene glycol, to form a hybridoma cell (Goding, Monoclonal Antibodies: Principles and Practice, Academic Press, (1986) pp. 59-103). Immortalized cell lines are usually transformed mammalian cells, particularly myeloma cells of rodent, bovine and human origin. Usually, rat or mouse myeloma cell lines are employed. The hybridoma cells may be cultured in a suitable culture medium that preferably contains one or more substances that inhibit the growth or survival of the unfused, immortalized cells. For example, if the parental cells lack the enzyme hypoxanthine guanine phosphoribosyl transferase (HGPRT or HPRT), the culture medium for the hybridomas typically will include hypoxanthine, aminopterin, and thymidine ("HAT medium"), which substances prevent the growth of HGPRT-deficient cells.

#### C. METHODS OF SCREENING MOLECULES THAT BIND TO THE HEREGULIN BINDING SITE IN HER3

The invention also encompasses methods of screening molecules to identify those which can act as agonists or antagonists of the heregulin/HER3 interaction. Such molecules may comprise small molecules or polypeptides, including antibodies. Examples of small molecules include, but are not limited to, small peptides or peptide-like molecules, preferably soluble peptides, and synthetic non-peptidyl organic or inorganic compounds. The screening assays for drug candidates are designed to identify compounds or molecules that bind or complex with the ligand or receptor polypeptides identified herein, or otherwise interfere with the interaction of these polypeptides with other cellular proteins. Such screening assays will include assays amenable to high-

include traditional approaches, such as, e.g., cross-linking, co-immunoprecipitation, and co-purification through gradients or chromatographic columns. In addition, protein-protein interactions can be monitored by using a yeast-based genetic system described by Fields and co-workers (Fields and Song, *Nature (London)*, 340:245-246 (1989); Chien et al., *Proc. Natl. Acad. Sci. USA*, 88:9578-9582 (1991)) as disclosed by Chevray and Nathans, *Proc. Natl. Acad. Sci. USA*, 89: 5789-5793 (1991). Many transcriptional activators, such as yeast GAL4, consist of two physically discrete modular domains, one acting as the DNA-binding domain, the other one functioning as the transcription-activation domain. The yeast expression system described in the foregoing publications (generally referred to as the "two-hybrid system") takes advantage of this property, and employs two hybrid proteins, one in which the target protein is fused to the DNA-binding domain of GAL4, and another, in which candidate activating proteins are fused to the activation domain. The expression of a GAL1-*lacZ* reporter gene under control of a GAL4-activated promoter depends on reconstitution of GAL4 activity via protein-protein interaction. Colonies containing interacting polypeptides are detected with a chromogenic substrate for β-galactosidase. A complete kit (MATCHMAKER™) for identifying protein-protein interactions between two specific proteins using the two-hybrid technique is commercially available from Clontech. This system can also be extended to map protein domains involved in specific protein interactions as well as to pinpoint amino acid residues that are crucial for these interactions.

Compounds or molecules that interfere with the interaction of a ligand or receptor polypeptide identified herein and other intra- or extracellular components can be tested as follows: usually a reaction mixture is prepared containing the product of the gene and the intra- or extracellular component under conditions and for a time allowing for the interaction and binding of the two products. To test the ability of a candidate compound to inhibit binding, the reaction is run in the absence and in the presence of the test compound. In addition, a placebo may be added to a third reaction mixture, to serve as positive control. The binding (complex formation) between the test compound

and the intra- or extracellular component present in the mixture is monitored as described hereinabove. The formation of a complex in the control reaction(s) but not in the reaction mixture containing the test compound indicates that the test compound interferes with the interaction of the test compound and its reaction partner.

5 To assay for antagonists, the ligand or receptor polypeptide may be added to a cell along with the compound to be screened for a particular activity and the ability of the compound to inhibit the activity of interest in the presence of the ligand or receptor polypeptide indicates that the compound is an antagonist to the ligand or receptor polypeptide. Alternatively, antagonists may be detected by combining the ligand or  
10 receptor polypeptide and a potential antagonist with membrane-bound polypeptide receptors or recombinant receptors under appropriate conditions for a competitive inhibition assay. The ligand or receptor polypeptide can be labeled, such as by radioactivity, such that the number of polypeptide molecules bound to the receptor can be used to determine the effectiveness of the potential antagonist. The gene encoding  
15 the receptor can be identified by numerous methods known to those of skill in the art, for example, ligand panning and FACS sorting. Coligan et al., Current Protocols in Immun., 1(2): Chapter 5 (1991). Preferably, expression cloning is employed wherein polyadenylated RNA is prepared from a cell responsive to the ligand or receptor polypeptide and a cDNA library created from this RNA is divided into pools and used to  
20 transfect COS cells or other cells that are not responsive to the ligand or receptor polypeptide. Transfected cells that are grown on glass slides are exposed to labeled ligand or receptor polypeptide. The ligand or receptor polypeptide can be labeled by a variety of means including iodination or inclusion of a recognition site for a site-specific protein kinase. Following fixation and incubation, the slides are subjected to  
25 autoradiographic analysis. Positive pools are identified and sub-pools are prepared and re-transfected using an interactive sub-pooling and re-screening process, eventually yielding a single clone that encodes the putative receptor.

As an alternative approach, labeled ligand polypeptide can be photoaffinity-linked with cell membrane or extract preparations that express receptor molecule. Cross-linked material is resolved by PAGE and exposed to X-ray film. The labeled complex containing the receptor can be excised, resolved into peptide fragments, and subjected to 5 protein micro-sequencing. The amino acid sequence obtained from micro-sequencing would be used to design a set of degenerate oligonucleotide probes to screen a cDNA library to identify the gene encoding the putative receptor.

A representative embodiment of this invention comprises a method of screening for a molecule that interacts with the heregulin binding domain in HER3 ("HER3 10 heregulin binding domain") comprising the steps of contacting a population of molecules with a polypeptide comprising the HER3 heregulin binding domain, allowing the population of molecules and the HER3 heregulin binding domain to interact under conditions that facilitate an interaction, determining the presence of a molecule that interacts with the HER3 heregulin binding domain, and then separating molecules that 15 do not interact with the HER3 heregulin binding domain from molecules that do. In a specific embodiment, the method further comprises purifying a molecule that interacts with the HER3 heregulin binding domain. The identified molecule can be used to modulate a function performed by HER3. This embodiment of the invention is well suited to screen chemical libraries for molecules which modulate, e.g., inhibit, antagonize, 20 or agonize or mimic, the activity of HER3 as measured by one of the assays disclosed herein or known in the art (see, e.g. Aigular et al., (1999) Oncogene 18, 6050-6062). The chemical libraries can be peptide libraries, peptidomimetic libraries, chemically synthesized libraries, recombinant, e.g., phage display libraries, and in vitro translation-based libraries, other non-peptide synthetic organic libraries.

25 Exemplary libraries are commercially available from several sources (ArQule, Tripos/PanLabs, ChemDesign, Pharmacopoeia). In some cases, these chemical libraries are generated using combinatorial strategies that encode the identity of each member of the library on a substrate to which the member compound is attached, thus allowing

direct and immediate identification of a molecule that is an effective modulator. Thus, in many combinatorial approaches, the position on a plate of a compound specifies that compound's composition. Also, in one example, a single plate position may have from 1-20 chemicals that can be screened by administration to a well containing the interactions of interest. Thus, if modulation is detected, smaller and smaller pools of interacting pairs can be assayed for the modulation activity. By such methods, many candidate molecules can be screened.

Many diversity libraries suitable for use are known in the art and can be used to provide compounds to be tested according to the present invention. Alternatively, 10 libraries can be constructed using standard methods. Chemical (synthetic) libraries, recombinant expression libraries, or polysome-based libraries are exemplary types of libraries that can be used.

In one embodiment, one can screen peptide libraries to identify molecules that interact with HER3 heregulin binding domain protein sequences. In such methods, 15 peptides that bind to the HER3 heregulin binding domain are identified by screening libraries that encode a random or controlled collection of amino acids. Peptides encoded by the libraries are expressed as fusion proteins of bacteriophage coat proteins, the bacteriophage particles are then screened against the protein of interest.

Accordingly, peptides having a wide variety of uses, such as therapeutic, 20 prognostic or diagnostic reagents, are thus identified without any prior information on the structure of the expected ligand or receptor molecule. Typical peptide libraries and screening methods that can be used to identify molecules that interact with HER3 heregulin binding domain sequences are disclosed for example in U.S. Patent Nos. 5,723,286 issued 3 March 1998 and 5,733,731 issued 31 March 1998.

25 Small molecules and ligands that interact with HER3 heregulin binding domain can be identified through related embodiments of such screening assays. For example, small molecules can be identified that interfere with protein function, including molecules that interfere with the tyrosine kinase activity associated with HER3.

A typical embodiment is a method of identifying a compound which specifically binds the HER3 heregulin binding domain, comprising the steps of contacting the HER3 heregulin binding domain with a test compound under conditions favorable to binding; and then determining whether said test compound binds to said HER3 heregulin binding  
5 domain so that a compound which binds to said domain can be identified. As the interaction between various receptor tyrosine kinases and a variety of test compounds have been previously described, skilled artisans are familiar with the conditions conducive to binding. A specific embodiment of this aspect of the invention includes the steps of transfecting cells with a construct encoding HER3, contacting said cells with  
10 said test compound that is tagged or labelled with a detectable marker and then analyzing said cells for the presence bound test compound. In contexts where the transfected cells are observed to preferentially bind the test compound as compared to cells that have not been transfected with a HER3 construct, this indicates that the test compounds is binding to the HER3 protein expressed by those cells.

15       A test compound which binds said HER3 heregulin binding domain may then be further screened for the inhibition of a biological activity (e.g. tyrosine kinase activity) associated with HER3. Such an embodiment includes, for example determining whether said test compound inhibits the tyrosine kinase activity associated with HER3 by utilizing molecular biological protocols to create recombinant contracts whose enzymological and  
20 biological properties can be examined directly. Enzymology is performed for example, by measuring tyrosine kinase activity in vitro or in HER3 expressing cells using standard assays.

#### D. ASSAY METHODS

25       Diagnostic methods are also provided herein. For instance, the antagonists or agonists may be employed to detect HER3 and/or heregulin in mammals known to be or suspected of having a HER3 and/or heregulin related pathological condition. The antagonist or agonist molecule may be used, e.g., in immunoassays to detect or quantitate

heregulin and/or HER3 in a sample. A sample, such as cells obtained from a mammal, can be incubated in the presence of a labeled antagonist or agonist molecule, and detection of the labeled antagonist or agonist bound in the sample can be performed. Such assays, including various clinical assay procedures, are known in the art, for instance 5 as described in Voller et al., Immunoassays, University Park, 1981.

Ligand/receptor binding studies may be carried out in any known assay method, such as competitive binding assays, direct and indirect sandwich assays, and immunoprecipitation assays. Cell-based assays and animal models can be used as diagnostic methods and to further understand the interaction between the ligands and 10 receptors identified herein and the development and pathogenesis of the conditions and diseases referred to herein.

In one approach, mammalian cells may be transfected with the ligands or receptors described herein, and the ability of the agonists or antagonists to stimulate or inhibit binding or activity is analyzed. Suitable cells can be transfected with the desired 15 gene, and monitored for activity. Such transfected cell lines can then be used to test the ability of antagonist(s) or agonist(s) to inhibit or stimulate, for example, to modulate B-cell proliferation or Ig secretion. Cells transfected with the coding sequence of the genes identified herein can further be used to identify drug candidates for the treatment of immune related diseases or cancer.

20 In addition, primary cultures derived from transgenic animals can be used in the cell-based assays. Techniques to derive continuous cell lines from transgenic animals are well known in the art (see, e.g., Small et al., Mol. Cell. Biol., 5:642-648 (1985)).

#### E. FORMULATIONS

25 The HER3 antagonists or agonists described herein, are optionally employed in a carrier. Suitable carriers and their formulations are described in Remington's Pharmaceutical Sciences, 16th ed., 1980, Mack Publishing Co., edited by Osol et al. Typically, an appropriate amount of a pharmaceutically-acceptable salt is used in the carrier

to render the formulation isotonic. Examples of the carrier include saline, Ringer's solution and dextrose solution. The pH of the carrier is preferably from about 5 to about 8, and more preferably from about 7.4 to about 7.8. It will be apparent to those persons skilled in the art that certain carriers may be more preferable depending upon, for instance, the route 5 of administration and concentration of active agent being administered. The carrier may be in the form of a lyophilized formulation or aqueous solution.

Acceptable carriers, excipients, or stabilizers are preferably nontoxic to cells and/or recipients at the dosages and concentrations employed, and include buffers such as phosphate, citrate, and other organic acids; antioxidants including ascorbic acid and 10 methionine; preservatives (such as octadecyldimethylbenzyl ammonium chloride; hexamethonium chloride; benzalkonium chloride, benzethonium chloride; phenol, butyl or benzyl alcohol; alkyl parabens such as methyl or propyl paraben; catechol; resorcinol; cyclohexanol; 3-pentanol; and m-cresol); low molecular weight (less than about 10 residues) polypeptides; proteins, such as serum albumin, gelatin, or immunoglobulins; 15 hydrophilic polymers such as polyvinylpyrrolidone; amino acids such as glycine, glutamine, asparagine, histidine, arginine, or lysine; monosaccharides, disaccharides, and other carbohydrates including glucose, mannose, or dextrans; chelating agents such as EDTA; sugars such as sucrose, mannitol, trehalose or sorbitol; salt-forming counter-ions such as sodium; and/or non-ionic surfactants such as TWEEN™, PLURONICS™ or 20 polyethylene glycol (PEG).

The formulation may also contain more than one active compound as necessary for the particular indication being treated, preferably those with complementary activities that do not adversely affect each other.

The HER3 antagonist or agonist described herein, may also be entrapped in 25 microcapsules prepared, for example, by coacervation techniques or by interfacial polymerization, for example, hydroxymethylcellulose or gelatin-microcapsules and poly-(methylmethacrylate) microcapsules, respectively, in colloidal drug delivery systems (for example, liposomes, albumin microspheres, microemulsions, nano-particles and

nanocapsules) or in macroemulsions. Such techniques are disclosed in Remington's Pharmaceutical Sciences 16th edition, Osol, A. Ed. (1980).

The formulations to be used for *in vivo* administration should be sterile. This is readily accomplished by filtration through sterile filtration membranes. Sustained-release preparations may be prepared. Suitable examples of sustained-release preparations include semipermeable matrices of solid hydrophobic polymers containing the active agent, which matrices are in the form of shaped articles, *e.g.* films, or microcapsules. Examples of sustained-release matrices include polyesters, hydrogels (for example, poly(2-hydroxyethyl-methacrylate), or poly(vinylalcohol)), polylactides (U.S. Pat. No. 10 3,773,919), copolymers of L-glutamic acid and  $\gamma$  ethyl-L-glutamate, non-degradable ethylene-vinyl acetate, degradable lactic acid-glycolic acid copolymers such as the LUPRON DEPOT<sup>TM</sup> (injectable microspheres composed of lactic acid-glycolic acid copolymer and leuprolide acetate), and poly-D-( $-$ )-3-hydroxybutyric acid. While polymers such as ethylene-vinyl acetate and lactic acid-glycolic acid enable release of 15 molecules for over 100 days, certain hydrogels release proteins for shorter time periods.

Related embodiments of the invention include methods for the preparation of a medicament for the treatment of pathological conditions including cancer by preparing a HER3 variant polypeptide (*e.g.* a variant having a substitution at V110) composition for administration to a mammal having the pathological condition. A related method is the 20 use of an effective amount of a HER3 variant polypeptide in the preparation of a medicament for the treatment of cancer. Such methods typically involve the steps of including an amount of HER3 variant polypeptide sufficient to inhibit the interaction of heregulin and HER3 *in vivo* and an appropriate amount of a physiologically acceptable carrier. As is known in the art, optionally other agents can be included in these 25 preparations.

F. MODES OF THERAPY

The invention further provides methods for modulating HER3, and/or heregulin activity in mammalian cells which comprise exposing the cells to a desired amount of antagonist or agonist that affects heregulin interaction with HER3. Preferably, the amount 5 of antagonist or agonist employed will be an amount effective to affect the binding and/or activity of the respective ligand or respective receptor to achieve a therapeutic effect. This can be accomplished *in vivo* or *ex vivo* in accordance, for instance, with the methods described below. Exemplary conditions or disorders to be treated with HER3 agonists or heregulin agonists include cancer such as those associated with an aberrant expression of 10 HER3 and/or heregulin.

The molecules described herein are useful in treating various pathological conditions, such as cancer. These conditions can be treated by stimulating or inhibiting a selected activity associated with heregulin or HER3 in a mammal through, for example, administration of one or more antagonists or agonists described herein.

15 Diagnosis in mammals of the various pathological conditions described herein can be made by the skilled practitioner. Diagnostic techniques are available in the art which allow, e.g., for the diagnosis or detection of cancer or immune related disease in a mammal. For instance, cancers may be identified through techniques, including but not limited to, palpation, blood analysis, x-ray, NMR and the like.

20 The antagonist(s) or agonist(s) can be administered in accord with known methods, such as intravenous administration as a bolus or by continuous infusion over a period of time, by intramuscular, intraperitoneal, intracerebrospinal, subcutaneous, intra-articular, intrasynovial, intrathecal, oral, topical, or inhalation routes. Optionally, administration may be performed through mini-pump infusion using various 25 commercially available devices. The antagonists or agonists may also be employed using gene therapy techniques which have been described in the art. Effective dosages and schedules for administering antagonists or agonists may be determined empirically, and making such determinations is within the skill in the art. Single or multiple dosages may be

employed. It is presently believed that an effective dosage or amount of antagonist or agonist used alone may range from about 1 ng/kg to about 100 mg/kg of body weight or more per day. Interspecies scaling of dosages can be performed in a manner known in the art, e.g., as disclosed in Mordenti et al., Pharmaceut. Res., 8:1351 (1991).

5 As noted above, the HER3 polypeptides, HER3 polypeptide variants, HER3 polypeptide fragments, HER3 polynucleotides encoding said polypeptides, variants and fragments, and the HER3 agents useful in the methods of the invention can be incorporated into pharmaceutical compositions suitable for administration into a mammal. Such compositions typically comprise at least one HER3 polypeptide, HER3 10 polypeptide variant, HER3 polypeptide fragment, HER3 polynucleotide encoding said polypeptide, variant or fragment, an HER3 agent, or a combination thereof, and a pharmaceutically acceptable carrier. Methods for formulating the HER3 compounds of the invention for pharmaceutical administration are known to those of skill in the art. See, for example, Remington: The Science and Practice of Pharmacy, 19<sup>th</sup> Edition, 15 Gennaro (ed.) 1995; Mack Publishing Company, Easton, PA.

As used herein the language "pharmaceutically acceptable carrier" is intended to include any and all solvents, dispersion media, coatings, antibacterial and antifungal agents, isotonic and absorption delaying agents, and the like, compatible with pharmaceutical administration. The use of such media and agents for pharmaceutically 20 active substances is well known in the art. Except insofar as any conventional media or agent is incompatible with the active compound, such media can be used in the compositions of the invention. Supplementary active compounds can also be incorporated into the compositions. A pharmaceutical composition of the, invention is formulated to be compatible with its intended route of administration.

25 The pharmaceutical compositions of the invention, comprising HER3 polypeptides, HER3 polypeptide variants, HER3 polypeptide fragments, polynucleotides encoding said HER3 polypeptides, variants and fragments, as well as HER3 agents, as defined above, are administered in therapeutically effective amounts. The

"therapeutically effective amount" refers to a nontoxic dosage level sufficient to induce a desired biological result (e.g. a diminution of the severity of the symptoms associated with a pathological condition such as breast or ovarian cancer). Amounts for administration may vary based upon the desired activity, the diseased state of the 5 mammal being treated, the dosage form, method of administration, patient factors such as age, sex, and severity of disease. It is recognized that a therapeutically effective amount is provided in a broad range of concentrations. Such range can be determined based on in vitro and/or in vivo assays.

Therapeutic compositions of the HER3 can be prepared by mixing the desired 10 HER3 molecule having the appropriate degree of purity with optional pharmaceutically acceptable carriers, excipients, or stabilizers (Remington's Pharmaceutical Sciences, 16th edition, Osol, A. ed. (1980)), in the form of lyophilized formulations, aqueous solutions or aqueous suspensions. Acceptable carriers, excipients, or stabilizers are preferably nontoxic to recipients at the dosages and concentrations employed, and include buffers 15 such as Tris, HEPES, PIPES, phosphate, citrate, and other organic acids; antioxidants including ascorbic acid and methionine; preservatives (such as octadecyldimethylbenzyl ammonium chloride; hexamethonium chloride; benzalkonium chloride, benzethonium chloride; phenol, butyl or benzyl alcohol; alkyl parabens such as methyl or propyl paraben; catechol; resorcinol; cyclohexanol; 3-pentanol; and m-cresol); low molecular 20 weight (less than about 10 residues) polypeptides; proteins, such as serum albumin, gelatin, or immunoglobulins; hydrophilic polymers such as polyvinylpyrrolidone; amino acids such as glycine, glutamine, asparagine, histidine, arginine, or lysine; monosaccharides, disaccharides, and other carbohydrates including glucose, mannose, or dextrins; sugars such as sucrose, mannitol, trehalose or sorbitol; salt-forming counter- 25 ions such as sodium; and/or non-ionic surfactants such as TWEEN™, PLURONIC™ or polyethylene glycol (PEG).

Additional examples of such carriers include ion exchangers, alumina, aluminum stearate, lecithin, serum proteins, such as human serum albumin, buffer substances such

as glycine, sorbic acid, potassium sorbate, partial glyceride mixtures of saturated vegetable fatty acids, water, salts, or electrolytes such as protamine sulfate, disodium hydrogen phosphate, potassium hydrogen phosphate, sodium chloride, colloidal silica, magnesium trisilicate, polyvinyl pyrrolidone, and cellulose-based substances. Carriers for topical or gel-based forms include polysaccharides such as sodium carboxymethylcellulose or methylcellulose, polyvinylpyrrolidone, polyacrylates, polyoxyethylene-polyoxypropylene-block polymers, polyethylene glycol, and wood wax alcohols. For all administrations, conventional depot forms are suitably used. Such forms include, for example, microcapsules, nano-capsules, liposomes, plasters, inhalation forms, nose sprays, sublingual tablets, and sustained-release preparations.

Solutions or suspensions used for administering HER3 can include the following components: a sterile diluent such as water for injection, saline solution; fixed oils, polyethylene glycols, glycerine, propylene glycol or other synthetic solvents; antibacterial agents such as benzyl alcohol or methyl parabens; antioxidants such as ascorbic acid or sodium bisulfite; chelating agents such as EDTA; buffers such as acetates, citrates or phosphates and agents for the adjustment of tonicity such as sodium chloride or dextrose. In one embodiment, a pharmaceutical composition can be delivered via slow release formulation or matrix comprising HER3 protein or DNA constructs suitable for expression of HER3 protein into or around a site within the body.

HER3 can also be administered in the form of a variety of sustained-release preparations. For example, HER3 may be delivered to the lung for slow release via encapsulation or carrier materials such as liposomes, or other drug "shells" such as albumin (Albunex by Molecular Biosystems), sugars (Levovist by Schering), gelatins, or lipids. Other suitable examples of sustained-release preparations for use with polypeptides including semipermeable matrices of solid hydrophobic polymers containing the protein, which matrices are in the form of shaped articles, e.g., films, or microcapsules. Examples of sustained-release matrices include polyesters, hydrogels (e.g., poly(2-hydroxyethyl-methacrylate) as described by Langer *et al.*, J. Biomed. Mater. Res.,

15: 167-277 (1981) and Langer, Chem. Tech., 12: 98-105 (1982) or poly(vinylalcohol)),  
polylactides (U.S. Patent No. 3,773,919, EP 58,481), copolymers of L-glutamic acid and  
gamma ethyl-L-glutamate (Sidman *et al.*, Biopolymers, 22: 547-556 (1983)), non-  
degradable ethylene-vinyl acetate (Langer *et al.*, *supra*), degradable lactic acid-glycolic acid  
5 copolymers such as the Lupron Depot (injectable microspheres composed of lactic acid-  
glycolic acid copolymer and leuprolide acetate), and poly-D-(*-*)-3-hydroxybutyric acid  
(EP 133,988).

The route of administration may vary depending on the desired effect and/or outcome. Generally for initiation of an HER3 mediated response, introduction of the  
10 HER3 at or near the desired site of response is utilized. Alternatively additional routes of administration, such as a systemic administration of HER3, may be employed. Examples of routes of administration include parenteral, e.g., intravenous, intradermal, intramuscular, subcutaneous, oral (e.g., inhalation) transdermal (topical), transmucosal (e.g. a nasal spray), and rectal administration. The HER3 polypeptide may also be  
15 administered by perfusion techniques, such as isolated tissue perfusion, to exert local therapeutic effects. Solutions or suspensions used for parenteral, intradermal, or subcutaneous application can include the following components: a sterile diluent such as water for injection, saline solution; fixed oils, polyethylene glycols, glycerine, propylene glycol or other synthetic solvents; antibacterial agents such as benzyl alcohol or methyl  
20 parabens; antioxidants such as ascorbic acid or sodium bisulfite; chelating agents such as EDTA; buffers such as acetates, citrates or phosphates and agents for the adjustment of tonicity such as sodium chloride or dextrose. Regimens of administration may vary. A single dose or multiple doses of the agent may be used. Such regimens can vary depending on the severity of the disease and the desired outcome. Following  
25 administration of a HER3 polypeptide to the mammal, the mammal's physiological condition can be monitored in various ways well known to the skilled practitioner familiar with the pathological condition to be treated (e.g. breast or ovarian cancer).

It is contemplated that yet additional therapies may be employed in the methods. The one or more other therapies may include but are not limited to, administration of radiation therapy, cytokine(s), growth inhibitory agent(s), chemotherapeutic agent(s), cytotoxic agent(s), tyrosine kinase inhibitors, ras farnesyl transferase inhibitors, angiogenesis 5 inhibitors, and cyclin-dependent kinase inhibitors which are known in the art and defined further with particularity in Section I above. In addition, therapies based on therapeutic antibodies that target tumor antigens such as Rituxan™ or Herceptin™ as well as anti-angiogenic antibodies such as anti-VEGF.

Preparation and dosing schedules for chemotherapeutic agents may be used 10 according to manufacturers' instructions or as determined empirically by the skilled practitioner. Preparation and dosing schedules for such chemotherapy are also described in *Chemotherapy Service* Ed., M.C. Perry, Williams & Wilkins, Baltimore, MD (1992). The chemotherapeutic agent may precede, or follow administration of, e.g. an antagonist, or may be given simultaneously therewith. The antagonist, for instance, may also be 15 combined with an anti-oestrogen compound such as tamoxifen or an anti-progesterone such as onapristone (see, EP 616812) in dosages known for such molecules.

#### G. ARTICLES OF MANUFACTURE

In another embodiment of the invention, an article of manufacture containing 20 materials useful for the treatment of the disorders described above is provided. The article of manufacture comprises a container and a label. Suitable containers include, for example, bottles, vials, syringes, and test tubes. The containers may be formed from a variety of materials such as glass or plastic. The container holds a composition which is effective for treating the condition and may have a sterile access port (for example the 25 container may be an intravenous solution bag or a vial having a stopper pierceable by a hypodermic injection needle). The active agents in the composition may comprise antagonist(s) or agonist(s). The label on, or associated with, the container indicates that the composition is used for treating the condition of choice. The article of manufacture

may further comprise a second container comprising a pharmaceutically-acceptable buffer, such as phosphate-buffered saline, Ringer's solution and dextrose solution. It may further include other materials desirable from a commercial and user standpoint, including other buffers, diluents, filters, needles, syringes, and package inserts with  
5 instructions for use.

#### H. BRIEF CHARACTERIZATION OF ASPECTS OF THE INVENTION

As disclosed herein, the predominant ligand binding site for heregulin is located  
10 in domain I of HER3. The disclosure identifies two proteolytic sites in HER3-ECD<sup>I-IV</sup> at positions 50 and 270. Cleavage at position 50 is fully protected by hrg binding, whereas position 270 is unprotected. Hrg protection at position 50 is a result of the specific interaction between hrg and HER3-ECD<sup>I-IV</sup>. Only two fragments were detected by SDS-PAGE and mass spectrometry. The N-terminal fragment generated from  
15 cleavage at residue 50 could not be detected, but its absence could be due to additional cleavage sites between residues 20 and 50. The cleavage site at position 270 is not protected by hrg, but the fragments generated by this cleavage are held together by disulfide bonds. Hrg protects only one of the cleavage sites in a ligand-specific manner. The extreme C terminus is also sensitive to proteolysis, which could be blocked by an  
20 antibody against the V-5 epitope tag but not by hrg.

This protection at position 50 can be explained in two different ways, which include binding of hrg at or near position 50 or indirect protection resulting from a conformational change in HER3-ECD<sup>I-IV</sup>, induced by hrg binding. In order to distinguish between these two possibilities based on the results of the proteolysis experiments; we expressed domain I and II of HER3-ECD<sup>I-IV</sup> (HER3-ECD<sup>I-II</sup>) and assayed it for binding.  
25

As illustrated in the Examples below, HER3-ECD<sup>I-II</sup> polypeptide is able to bind heregulin and the association state and binding properties of HER3-ECD<sup>I-II</sup> were

characterized. For example, HER3-ECD<sup>I-II</sup> is shown to be a monomer and remains in a monomeric state when hrg is present. This is in contrast to the HER3-ECD<sup>I-IV</sup> expressed in S2 cells in which hrg reverses oligomerization to form a monomer (see, e.g. Landgraf, R., and Eisenberg, D. (2000) Biochemistry 39, 8503-8511). As discussed 5 below we analyzed direct hrg binding to HER3-ECD<sup>I-II</sup> and qualitatively demonstrated specific binding to hrg binding by a pull-down assay. An equilibrium dissociation constant of 68 nM for hrg binding was calculated by surface plasmon resonance in which the trx-hrg was immobilized. HER3-ECD<sup>I-II</sup> has 30-fold lower binding affinity than 10 HER3-ECD<sup>I-IV</sup> (2.3 nM) but a 7-fold greater affinity for its ligand than the extracellular domain of EGFR (500 nM) (see, e.g. Kohda et al., (1993) J. Biol. Chem. 268, 1976-1981). Consequently the results demonstrate that domains I and II of HER3-ECD are sufficient for hrg binding, and the results from proteolysis protection provide evidence that a ligand binding site is located in domain I.

Limited proteolysis of HER3-ECD<sup>I-IV</sup> and the expression of HER3-ECD<sup>I-II</sup> 15 provides evidence that the extent to which different domains of the type I receptor tyrosine kinases (EGFR, HER2, HER3, and HER4) contribute to ligand binding may not be conserved among the members of this EGFR family. The data provides evidence that domain I of HER3-ECD<sup>I-IV</sup> contains a site involved in ligand binding, whereas in EGFR, multiple lines of evidence provide evidence that the ligand contacts both domain 20 I and III (see, e.g. Lax et al., (1989) EMBO J. 8, 421-427; Lax et al., (1991) Cell Regul. 2, 337-345; Wu et al., (1989) J. Biol. Chem. 264, 17469-17475; Summerfield et al., (1996) J. Biol. Chem. 271, 19656-19659; Wu et al., (1990) Proc. Natl. Acad. Sci. U. S. A. 87, 3151-3155) but that domain III alone is sufficient for ligand binding (see, e.g. Kohda et al., (1993) J. Biol. Chem. 268, 1976-1981). It is noteworthy that the K<sub>d</sub> for transforming 25 growth factor binding to the monomeric ECD of EGFR is 500 nM. Domain III of EGFR binds transforming growth factor with a K<sub>d</sub> of 1.3 μM. The K<sub>d</sub> of HER3-ECD<sup>I-IV</sup> is 1.9 nM, whereas the K<sub>d</sub> for HER3-ECD<sup>I-II</sup> is 68 nM. Therefore, despite a loss of binding compared with HER3-ECD<sup>I-IV</sup>, HER3-ECD<sup>I-II</sup> still retains a higher affinity for

- its ligand than the EGFR ECD. EGFR forms dimers in response to ligand binding (see, e.g. Hurwitz et al., (1991) *J. Biol. Chem.* 266, 22035-22043), whereas HER3-ECD<sup>I-IV</sup> expressed in S2 cells exists as an oligomer but, following ligand binding, forms a monomer (see, e.g. Landgraf, R., and Eisenberg, D. (2000) *Biochemistry* 39, 8503-8511).
- 5 This may be a reflection of the different modes of ligand binding behavior in both receptors. The data provides evidence that for HER3-ECD<sup>I-IV</sup> ligand binding occurs partly in domain I, whereas domain III may also contribute to binding; the relative contribution of domains I and II to binding appears to be shifted in favor of domain I, for HER3.
- 10 An understanding of the activity of type I RTKs has been hindered by the lack of a molecular structure for any member of the family. It has been proposed that the structure of type I RTK ECDs may be similar to the insulin growth factor-1 receptor (IGF-1R) (see, e.g. Jorissen et al., (2000) *Protein Sci.* 9, 310-324; Garrett et al., (1998) *Nature* 394, 395-399). The structure of the first three domains of IGF-1R was solved by 15 x-ray crystallography at 2.6-Å resolution (see, e.g. Garrett et al., (1998) *Nature* 394, 395-399). IGF-1R is a type II receptor tyrosine kinase and is a member of the tyrosine kinase superfamily, which includes the type I (EGFR/HER3) subfamily (see, e.g. Ullrich, A., and Schlessinger, J. (1990) *Cell* 61, 203-212). HER3 and IGF-1R have significant sequence identity in portions of the extracellular domain and have a similar domain 20 organization (see, e.g. Yarden, Y., and Ullrich, A. (1988) *Annu. Rev. Biochem.* 57, 443-478; Lax et al., (1988) *Mol. Cell. Biol.* 8, 1970-1978). In addition, this equivalence has been proposed based on homology modeling between HER3-ECD<sup>I-IV</sup> and the IGF-1R structure (see, e.g. Jorissen et al., (2000) *Protein Sci.* 9, 310-324). The IGF-1R structure contains three domains (L1, S1, and L2), which are equivalent to domains I, II, and III in 25 HER3-ECD<sup>I-IV</sup> (Table I). Domains L1 and L2 are similar in sequence in IGF-1R (25% identity and 41% similarity), as are domains I and III in HER3-ECD<sup>I-IV</sup> (30% identity and 41% similarity). Domains L1 and L2 also have highly similar structures (see, e.g. Garrett et al., (1998) *Nature* 394, 395-399). Alanine-scanning mutagenesis of the insulin

receptor, a receptor closely related to IGF-1R, identified four regions in the primary sequence of L1 that were important for ligand binding (see, e.g. Williams et al., (1995) J. Biol. Chem. 270, 3012-3016). These regions map to the IGF-1R structure, which reveals a "hormone binding footprint" lying on the face of domain L1 (see, e.g. Garrett et al., 5 (1998) Nature 394, 395-399). A naturally occurring mutation in the insulin receptor at residue 58 lies within the footprint (see, e.g. van der Vorm et al., (1992) J. Biol. Chem. 267, 66-71). Other mutations in the insulin receptor also provide evidence minor contributions in domain L2 (see, e.g. Nakae et al., (1995) J. Biol. Chem. 270, 22017-22022). A model has been suggested in which ligand binding in the IGF-1R involves 10 domains L1 and L2 (see, e.g. Jorissen et al., (2000) Protein Sci. 9, 310-324), which correspond to domains I and III in HER3-ECD<sup>I-IV</sup>.

Our current analysis provides biochemical evidence for the proposed structural similarity of type I (HER3) and II (IGF-1R and IR) RTKs. First, a sequence alignment 15 of domain L1 in the IR and the IGF-1R and domain I in HER3-ECD<sup>I-IV</sup> was performed (Fig. 8). The cleavage site at position 50, which is protected by hrg corresponds to the putative ligand-binding region in domain L1 in IGF-1R (Fig. 9A). Residue 17 in IGF-1R (position 50 in HER3-ECD<sup>I-IV</sup>) lies on the same face as the binding footprint and is 11 Å away from residue 10, which lies within the binding footprint. Second, we expressed 20 HER3-ECD<sup>I-II</sup>, which binds hrg with a 68 nM dissociation constant, supporting the involvement of domain I in ligand binding. Third, the second proteolysis site in which the fragments are held together by a disulfide bridge at position 270 is located in the 25 cysteine-rich domain II of HER3-ECD<sup>I-IV</sup> (position 237 in the IGF-1R). Based on the IGF-1R structure, cleavage at this position is expected to result in a disulfide-linked fragment (Fig. 9B). Fourth, the large accessible surface area (1782 Å<sup>2</sup>) of domain L1 that is buried by domain S1 of IGF-1R and the conserved contacts in this interface in the type 1 RTKs (see, e.g. Jorissen et al., (2000) Protein Sci. 9, 310-324) provide evidence that these domains function as unit. This is consistent with our finding that the expression of domain I of HER3 requires the presence of domain II. Therefore, our

results are consistent with the proposal that HER3 has a structure similar to IGF-1R. These data provide further insight into areas in the HER3 receptor critical for heregulin binding.

The following examples are offered by way of illustration and not by way of limitation. Various citations are referenced throughout the specification (e.g. PCT application No. WO0160397A1). The disclosures of all citations in the specification are expressly incorporated herein by reference.

## EXAMPLES

10    Example 1: Common Materials

Soluble extracellular domains of recombinant HER3 (HER3-ECD<sup>I-IV</sup>) were purified from Chinese hamster ovary cells by the procedure of Kita et al. (see, e.g. Kita et al., (1996) Biochem. Biophys. Res. Commun. 226, 59-69). The 60-residue EGF-like domain of human heregulin  $\beta$ 1 (residues 177-237) (hrg), the binding-deficient heregulin mutant (NA5-hrg), and the thioredoxin-heregulin fusion with a C-terminal His and S-tag (trx-hrg) were generated and purified as described elsewhere (see, e.g. Landgraf et al., (1998) Biochemistry 37, 3220-3228). EGF was purchased from Sigma. Trypsin was purchased from Life Technologies, Inc. (catalog number 15400-013 and lot numbers 12P6334 and 1023241) and Sigma (catalog number T-1426 and lot number 70K7661), which had been treated with tosylamide-2-phenylethylchloromethyl ketone to remove chymotrypsin.

Example 2: Proteolytic Examination of Heregulin and HER3 Interaction

## A. Proteolytic Digestion.

5.0 µl of (HER3-ECD<sup>I-IV</sup>) at 2.3 mg/ml in storage buffer (20 mM sodium acetate pH 5.5 and 40 mM sodium chloride), 10 µl of phosphate-buffered saline (PBS): 136 mM NaCl, 2.68 mM KCl, 9.289 mM Na<sub>2</sub>HPO<sub>4</sub>, 1.969 mM KH<sub>2</sub>PO<sub>4</sub>), and 3.0 µl of trypsin (Life Technologies, Inc.; catalog number 15400-013 and lot number 12P6334) at 5.0 mg/ml in 5.0 mM EDTA and 150 mM sodium chloride were incubated at 37 °C for 1 h. Digestion was stopped by adding phenylmethylsulfonyl fluoride at a final concentration of 1.0 mM. 5.0 µl of the digested sample were added to 5.0 µl of 2× SDS-PAGE sample 10 buffer with and without 1.0 mM β-mercaptoethanol (β-ME). These samples were then analyzed by SDS-PAGE on a 10-15% Phast gel (Amersham Pharmacia Biotech) and visualized by Coomassie staining. Different sources of trypsin were compared, but the best results were obtained with tissue culture grade trypsin from Life Technologies, Inc., which may contain other proteases.

15 HER3-ECD<sup>I-IV</sup> was titrated with hrg to determine the minimum amount required for complete protection of HER3-ECD<sup>I-IV</sup> from proteolysis. Hrg in PBS was incubated with HER3-ECD<sup>I-IV</sup> for 30 min at room temperature, followed by trypsin as described above. The final concentration of hrg in the digest ranged from 1.7 to 17 µM with a constant HER3-ECD<sup>I-IV</sup> concentration of 7.7 µM. These reaction mixtures were 20 analyzed by reducing SDS-PAGE as described above. The proteolytic digest was repeated with NA<sub>5</sub>-hrg and EGF using a molar ratio of 1:1.

B. Proteolysis of HER3-ECD<sup>I-IV</sup> Results in Two Primary Fragments That Remain Associated under Oxidizing Conditions.

First, we digested HER3-ECD<sup>I-IV</sup> to learn if limited proteolysis leads to a 25 fragment still capable of binding hrg. This results in two principal fragments (Fig. 1A, lane 3) with apparent molecular masses of 24 kDa (fragment 1) and 54 kDa (fragment 2), compared with an apparent molecular mass for the nonproteolyzed HER3-ECD<sup>I-IV</sup> of 80 kDa. Gel electrophoresis of the proteolyzed HER3-ECD<sup>I-IV</sup> in the absence of a

reducing agent shows that the two fragments remain linked by disulfide bonds (Fig. 1A, lane 4). Fragment 2 was identified as the C-terminal fragment by Western blot using the V5 C-terminal epitope-tagged HER3-ECD<sup>I-IV</sup> (Fig. 1B).

5 C. Proteolytic Cleavage of HER3-ECD<sup>I-IV</sup> Abolishes Herregulin Binding

To determine the effects of cleavage of HER3-ECD<sup>I-IV</sup> on its ability to bind hrg, a gel mobility shift assay was performed. In a native gel analysis, proteolyzed HER3-ECD<sup>I-IV</sup> (Fig. 2A, lane 2) shows an electrophoretic mobility similar to the nonproteolyzed HER3-ECD<sup>I-IV</sup> (Fig. 2A, lane 1). The nonproteolyzed HER3-ECD<sup>I-IV</sup> 10 shows a discrete shift in the presence of a 1:1 molar ratio of hrg to HER3-ECD<sup>I-IV</sup> (Fig. 2A, lane 3). The proteolyzed HER3-ECD<sup>I-IV</sup> does not shift its gel position in the presence of hrg (Fig. 2A, lane 4). This finding provide evidences that the two disulfide-linked fragments, generated by proteolytic digestion (Fig. 1A, lane 4) do not bind hrg.

To check whether the proteolyzed HER3-ECD<sup>I-IV</sup> binds hrg, we used a pull-down assay under physiological salt conditions. In this analysis, we measured the HER3-ECD<sup>I-IV</sup> and proteolyzed HER3-ECD<sup>I-IV</sup> that could be dissociated from immobilized thioredoxin-hereregulin (trx-hrg) by the addition of hrg. Using trx-hrg-coupled S-protein agarose, HER3-ECD<sup>I-IV</sup> was eluted with hrg (Fig. 2B, lane 3). No proteolyzed HER3-ECD<sup>I-IV</sup> could be recovered by either elution with hrg (Fig. 2B, lane 4) or detergent (Fig. 2B, lane 7). However, HER3-ECD<sup>I-IV</sup> that was protected by hrg during proteolytic digestion could be recovered by detergent and competition with hrg (Fig. 2B, lanes 5 and 8). In addition, a trypsin preparation that was treated with phenylmethylsulfonyl fluoride prior to the addition to the digestion reaction did not abolish hrg binding by HER3-ECD<sup>I-IV</sup> (Fig. 2B, lane 9). Based on these results, we conclude that the proteolyzed 20 HER3-ECD<sup>I-IV</sup> does not bind hrg.

D. Heregulin Protects HER3-ECD<sup>I-IV</sup> from Proteolytic Cleavage

The observation that proteolytic cleavage destroys the binding of HER3-ECD<sup>I-IV</sup> to hrg provide evidences that cleavage occurs at or near the binding site. This led us to investigate the proteolysis of HER3-ECD<sup>I-IV</sup> in the presence of hrg. The digestion of 5 HER3-ECD<sup>I-IV</sup> in the presence of a molar excess of hrg (Fig. 3A, lane 3) produces a different cleavage pattern than in the absence of hrg (Fig. 3A, lane 2). Complete protection of HER3-ECD<sup>I-IV</sup> was observed (Fig. 3B, lane 3) at an approximate 1:1 molar ratio of hrg over HER3-ECD<sup>I-IV</sup>. Fragment 1 has a higher molecular weight in the presence of hrg (Fig. 3A, lane 3) than in its absence (Fig. 3A, lane 2), whereas the size of 10 fragment 2 is apparently not affected by the presence of hrg (Fig. 3A, lanes 2-5). The cleavage that produces fragment 1 is apparently blocked by hrg but produces an additional, smaller fragment, which was not detected in the SDS gel of the digest of HER3-ECD<sup>I-IV</sup>. These data provide evidence that hrg protects HER3-ECD<sup>I-IV</sup> from the proteolytic cleavage that generates fragment 1.

15

E. Localization of the Cleavage Sites

Taken together, these results provide evidence that there are at least two cleavage sites, one that is protected by hrg and another that is not. To localize the positions of the cleavage sites, we used N-terminal sequencing and mass spectrometry. Fragment 1 begins 20 at position 51 in the absence of hrg and at position 20 (the first residue in native HER3-ECD<sup>I-IV</sup>) in the presence of hrg (Fig. 4). The N-terminal 31-residue fragment generated from the cleavage following residue 50 was not detected by SDS-PAGE or by mass spectrometry. The corresponding size by mass spectrometry of fragment 1 is 26.7 kDa in the absence of hrg and 30 kDa in the presence (Fig. 4). The difference in mass of 25 fragment 1 in the presence and absence of hrg is 3270 daltons, which corresponds to the predicted size of 3216 daltons, based on the amino acid sequence of the missing fragment (Fig. 4). This provide evidences that the amino acid residues between positions

20 and 50 of HER3-ECD<sup>I-IV</sup> are not glycosylated. Based on these results, we conclude that hrg protects HER3-ECD<sup>I-IV</sup> from proteolysis at position 50.

The cleavage that generates fragment 2 was not blocked by hrg in our assays and is located at position 270. The mass of fragment 2 is 47.0 kDa both in the presence and 5 absence of hrg. The C terminus may contain additional cleavage sites that are not protected by hrg, because there is a missing C-terminal fragment (7.5 kDa) both in the presence and absence of hrg (Fig. 4). However, cleavage of the C terminus could be blocked by an antibody against the V5 epitope tag (Fig. 1b).

The cleavage sites on the carboxyl side of Tyr<sup>50</sup> and Phe<sup>270</sup> are more typical of 10 chymotryptic rather than tryptic cleavage. This cleavage could be due to chymotrypsin present in the solution purchased from Life Technologies, Inc. However, the identity of the specific protease involved does not affect the finding that hrg protects HER3-ECD<sup>I-IV</sup> from cleavage at Tyr<sup>50</sup> but not at Phe<sup>270</sup>.

15 F. Protection Is Due to a Specific Interaction of hrg with HER3-ECD<sup>I-IV</sup>

To show that hrg protection is a result of the specific interaction between hrg and HER3-ECD<sup>I-IV</sup>, we repeated the digestion in the presence of a binding-deficient mutant of hrg (NA<sub>5</sub>-hrg) and EGF. NA<sub>5</sub>-hrg is deficient in binding due to the fact that the N-terminal residues that confer specific HER3-ECD<sup>I-IV</sup> binding were mutated to 20 alanine. NA<sub>5</sub>-hrg and EGF do not shift the position of HER3-ECD<sup>I-IV</sup> in a gel mobility shift assay (Fig. 5, lanes 3 and 4). The cleavage pattern obtained in the presence of NA<sub>5</sub>-hrg and EGF matches that of unprotected HER3-ECD<sup>I-IV</sup> (Fig. 3A, lanes 4 and 5 versus lane 2), further confirming that they do not bind hrg. Therefore, the difference in the 25 cleavage pattern in the presence of hrg is due to a specific interaction between hrg and HER3-ECD<sup>I-IV</sup>.

Example 3: Heregulin Binding Assays

## A. Gel Mobility Shift Assays

HER3-ECD<sup>I-IV</sup> (1.4 µl) in storage buffer was incubated with 9.6 µl of hrg (6.6 µM in PBS) for 30 min at room temperature. 4.0-µl aliquots of this mixture were run on a 10-15% Phast gel under native conditions. This assay was repeated for NA<sub>5</sub>-hrg, EGF, and proteolyzed HER3-ECD<sup>I-IV</sup> under the same conditions.

## B. "Pull-down" Assay Using Immobilized Trx-hrg

The ability of proteolyzed HER3-ECD<sup>I-IV</sup> to bind hrg was analyzed by a pull-down assay. S-protein agarose (0.5 ml) (Novagen) was spun down and resuspended three times in PBS. Aliquots of 100 µl were spun again, and trx-hrg (600 nM) and bovine serum albumin (BSA) (1 mg/ml) were added to the resin. After incubating 15 min at 4 °C, the resin was washed five times with PBS, and blocked again with BSA. The following samples were added to each aliquot of resin: HER3-ECD<sup>I-IV</sup>, proteolyzed HER3-ECD<sup>I-IV</sup>, HER3-ECD<sup>I-IV</sup> protected by hrg during proteolysis, and HER3-ECD<sup>I-IV</sup> incubated with trypsin deactivated with 1 mM phenylmethylsulfonyl fluoride prior to the proteolysis reaction. HER3-ECD<sup>I-IV</sup> was at 600 nM. BSA (1 mg/ml) was also added to each aliquot. Following a 15-min incubation at 4 °C, the resin was spun down and resuspended three times in PBS. Each aliquot of resin was then resuspended in PBS containing 1 µM hrg or PBS. After a 1-min incubation at 4 °C, the samples were spun down, and the supernatant was diluted with 2× SDS-PAGE sample buffer containing 1.0 mM β-ME. Each aliquot of resin was then resuspended in SDS-PAGE sample buffer. The samples were analyzed by SDS-PAGE on a 4-15% polyacrylamide gradient gel (Bio-Rad) and visualized by Western blotting and chemiluminescence using a polyclonal antibody directed against the HER3-ECD (E38530; Transduction Laboratories, Lexington, KY) followed by incubation with a secondary antibody conjugated to horseradish peroxidase (Invitrogen).

The ability of HER3-ECD<sup>I-II</sup> to bind hrg was also evaluated in the pull-down assay in the same way, with the following exceptions. HER3-ECD<sup>I-II</sup> (600 nM) was added to the trx-hrx and control resins, and the individual samples were resuspended in the various competing ligands indicated in Fig. 5. HER3-ECD<sup>I-II</sup> was visualized by 5 Western blotting and chemiluminescence using a monoclonal antibody directed against the V-5 epitope conjugated to horseradish peroxidase (Invitrogen).

Example 4: Identification of the Digested Fragments and Amino Acid Analysis

Proteolytically digested HER3-ECD<sup>I-IV</sup> was diluted with sample buffer 10 containing β-ME and run on a 4-15% polyacrylamide gradient gel (Bio-Rad) as described above. The samples were then transferred to a polyvinylidene difluoride membrane, and the fragments were visualized by Ponceau stain. Edman sequencing of excised bands was performed in the UCLA Protein Microsequencing Facility using a Porton-Beckman Gas Phase Sequencer, and the first 10 residues of each fragment were identified. HER3-ECD<sup>I-IV</sup> was proteolyzed in the presence of an equimolar amount of an antibody against 15 the C-terminal V5 epitope for 30, 60, and 120 min. The C-terminal fragment was identified by Western blotting using an antibody against the C-terminal V5 epitope (Invitrogen).

For the amino acid analysis the molar concentrations of HER3-ECD<sup>I-IV</sup>, hrg, and 20 NA<sub>5</sub>-hrg were determined by amino acid analysis at the UCLA Protein Microsequencing Facility. The samples were hydrolyzed in 6 N HCl at 110 °C for 18 h under a vacuum in a nitrogen atmosphere. The hydrolysate was visualized with phenylisothiocyanate. The phenylisothiocyanate amino acids were analyzed on a reverse phase column (Novapak) using a sodium acetate-acetonitrile gradient (see, e.g. Bidlingmeyer et al., (1984) J. Chromatogr. 336, 93-104; Cohen, S. A., and Strydom, D. J. (1988) Anal. Biochem. 174, 25 1-16).

Example 5: Matrix-assisted Laser Desorption Ionization (MALDI) Mass Spectrometry

We used MALDI time-of-flight mass spectrometry to measure the molecular mass of proteolyzed HER3-ECD<sup>I-IV</sup> and HER3-ECD<sup>I-II</sup>. The mass spectrometry was performed on a Voyager RP machine (Perceptive Biosystems, Framingham, Massachusetts) with BSA as an internal standard. 0.3 µl of HER3-ECD<sup>I-IV</sup> (2.3 mg/ml) in 20 mM sodium acetate pH 5.5, 40 mM sodium chloride was mixed with 0.5 µl of 10 mg/ml sinapinic acid in 70% acetonitrile, 0.1% trifluoracetate and dried on the MALDI plate. The digests (plus and minus hrg) of HER3-ECD<sup>I-IV</sup> were prepared in the same way, except that the sample was taken directly from the digest reaction after 1 h of incubation time. The digests were run under oxidizing and reducing conditions. The digests were reduced by the addition of 1 mM dithiothreitol prior to mixing the sample with the matrix. The molecular mass of HER3-ECD<sup>I-II</sup> (12 µM) in PBS was measured under the same conditions.

15    Example 6: Sequence Alignments and Surface Area Calculations

An initial multiple sequence alignment was performed with the GCG program Pileup (Wisconsin Package, SeqLab, SeqWeb) using the Blossum 62 matrix (see, e.g. Henikoff, S., and Henikoff, J. G. (1992) Proc. Natl. Acad. Sci. U. S. A. 89, 10915-10919). Empirical observations of the N terminus were used in conjunction with the initial 20 alignment to produce a final alignment. We used the GCG program Bestfit (Wisconsin Package, SeqLab, SeqWeb) (see, e.g. Smith, T., and Waterman, M. (1981) Adv. Appl. Math. 2, 482-489) to find the segment of greatest similarity between two sequences. The amount of solvent-exposed surface area of domain L1 that is buried by domain S1 of IGF-1R was calculated using the GCG program Areaimol (see, e.g. Lee, B., and Richards, F. M. (1971) J. Mol. Biol. 55, 379-400; Collaborative Computational Project. 25 (1994) Acta Crystallogr. Sect. D 50, 760-763).

Example 7: Cloning and Expression of HER3-ECD<sup>I-II</sup>

The HER3 cDNA was amplified from the pJTH3 plasmid provided by Amgen, using the N-terminal primer (CTA GTC TCT AGA TCC GAG GTG GGC AAC TCT) (SEQ ID NO: 7) and C-terminal primer (TAC CGA TCT AGA TTT CGG ACA GAG ACC CCC) (SEQ ID NO: 8). Following amplification, HER3-ECD<sup>I-II</sup> (residues 20-329) was cloned into the *Bgl*II and *Xba*I site of the pMT/BiP/V5-His-A expression vector (Invitrogen, Carlsbad, CA). This vector contains an N-terminal *Drosophila* leader sequence and C-terminal His tag and V5 epitope tags. S2 cells were co-transfected with the vector containing HER3-ECD<sup>I-II</sup> and the pCoHYGRO vector (Invitrogen), which provides hygromycin resistance. We obtained a stable cell line after 3 weeks of selection with 300 µg/ml hygromycin (Invitrogen). Five liters of S2 medium (Sigma) with 1% fetal bovine serum were inoculated with S2 cells and grown to a cell density of 5 × 10<sup>6</sup> in a spinner flask (Bellco) and then induced for 3 days at room temperature with 500 µM CuSO<sub>4</sub>. HER3-ECD<sup>I-II</sup> was secreted to the medium. We used an ammonium sulfate cut (80%, w/v) to precipitate the protein from the medium. The pellet was resolubilized in 20 mM Tris, pH 7.9, 1.5 M NaCl, and 0.1% Tween 20. The solution was dialyzed into 20 mM Tris, pH 7.9, 0.5 M NaCl, and 5 mM imidazole and then purified on a 5-ml Amersham Pharmacia Biotech "HITRAP Chelating" column (Amersham Pharmacia Biotech), loaded with NiSO<sub>4</sub>. Following elution from the column, the protein was dialyzed into 20 mM sodium potassium phosphate, pH 10. The protein was loaded onto an anion exchange column (Bio-Rad), and pure HER3-ECD<sup>I-II</sup> was collected from the flow-through.

Example 8: Multiangle Light Scattering of HER3-ECD<sup>I-II</sup>

Size exclusion chromatography was performed using a Tosohas G3000SWXL column, followed by light scattering on a miniDAWN three-angle light-scattering instrument (Wyatt Technologies, Santa Barbara, CA). Data analysis was carried out using ASTRA software. HER3-ECD<sup>I-II</sup> (112 µM) in 20 mM Hepes, pH 7.5, was injected onto

the column at a flow rate of 0.4 ml/min. The refractive index increment ( $dn/dc$ ) for the protein portion of the HER3-ECD<sup>I-II</sup> in this buffer was assumed to be equal to that of monomeric BSA (Sigma) ( $dn/dc = 0.181 \text{ cm}^3/\text{g}$ ). The carbohydrate  $dn/dc$  contribution was estimated to be  $0.157 \text{ cm}^3/\text{g}$  and was integrated into the final  $dn/dc$  ( $0.180 \text{ cm}^3/\text{g}$ ) proportionally. Calculated extinction coefficients uncorrected for folding effect were used. The error to noise in the light scatter data is about  $\pm 3\%$ . An error in the assumed extinction coefficient would result in a proportional error in the calculated molecular mass.

10    Example 9: Ultracentrifugation of HER3-ECD<sup>I-II</sup>

Sedimentation equilibrium was performed at  $4^\circ\text{C}$  in a Beckman Optima XL-A analytical ultracentrifuge using absorption optics at 280 nm. A 12-mm path length six-sector cell was used to measure protein samples at initial  $A_{280}$  values of 0.15, 0.35, and 0.75. All samples were in PBS. Sedimentation equilibrium profiles were measured at 12,000 and 15,000 rpm. The data were initially fitted with a nonlinear least-squares exponential fit for a single ideal species using Origin (version 3.01). Since no concentration or speed dependence of the molecular weight was apparent, the Beckman global analysis software (the "multifit" option of the above mentioned software) was used to analyze all six scans simultaneously. A partial specific volume of 0.712 calculated from the amino acid composition and corrected to  $4^\circ\text{C}$  was used (see, e.g. Cohn, E. J., and Edsall, J. T. (1943) in Proteins, Amino Acids and Peptides as Ions and Dipolar Ions (Cohn, E. J., and Edsall, J. T., eds), pp. 370-381, Reinhold Publishing Corp., New York; Laue et al., (1992) in Analytical Ultracentrifugation in Biochemistry and Polymer Science (Harding, S. E. , Rowe, A. J. , and Horton, J. C., eds), pp. 90-125, The Royal Society of Chemistry, Cambridge, United Kingdom).

Example 10: Surface Plasmon Resonance Measurement of Binding between HER3-ECD<sup>I-II</sup> and the EGF-like Domain of Heregulin

Trx-hrg (13  $\mu$ M) in MES buffer (100 mM pH 6.0) was immobilized on a BIACore CM5 chip using standard N-hydroxysuccinimide/1-ethyl-3-(3-dimethylaminopropyl)carbodiimide amine-coupling chemistry. The surface of the chip was blocked with ethanolamine and could be regenerated with 5.0 M NaCl and washes with running buffer (PBS + surfactant). HER3-ECD<sup>I-II</sup> (filtered through a 0.1- $\mu$ m filter unit (Millipore, Bedford, MA)) in running buffer was applied to the chip at various concentrations (in triplicate) to determine the dissociation constant. Data were analyzed with the BIAevaluation software. Competition experiments were carried out using hrg at an equimolar concentration to HER3-ECD<sup>I-II</sup>.

Example 11: Recombinant HER3-ECD<sup>I-II</sup> Is Sufficient for Heregulin Binding

To show that a ligand binding site is located within domain I of HER3-ECD<sup>I-IV</sup>, we recombinantly expressed domain I of HER3 alone (HER3-ECD<sup>I</sup>) as well as domains I and II together (HER3-ECD<sup>I-II</sup>). Expression of domain I alone resulted in an improperly folded protein because of incorrect disulfide formation, so only HER3-ECD<sup>I-II</sup> could be evaluated for ligand binding. The recombinant form of HER3-ECD<sup>I-II</sup> (residues 20-329) was expressed in *Drosophila* S2 cells and purified from the medium. The protein was found to be pure by SDS-PAGE analysis with an apparent molecular mass of 40 kDa (Fig. 6A). We used MALDI mass spectrometry to estimate the carbohydrate content. The molecular mass of HER3-ECD<sup>I-II</sup> was found to be between 38.5 and 40 kDa, compared with a theoretical molecular mass of 37.7 kDa (Fig. 6B). This provide evidences that HER3-ECD<sup>I-II</sup> has a carbohydrate content between 3.3 and 5.7% by weight.

Size exclusion chromatography followed by multiangle light scattering was used to show that recombinant HER3-ECD<sup>I-II</sup> is a monomer. The concentration of HER3-ECD<sup>I-II</sup> at the time of light scattering was 1.8  $\mu$ M. At this concentration, a single peak was observed with a molecular mass of ~34 kDa (S.E. =  $\pm$ 4%) corresponding to

monomeric HER3-ECD<sup>I-II</sup> (Fig. 6C). There was a slight shift in the peak upon the addition of a 1.3-fold molar excess of hrg. The shifted peak has an estimated molecular mass of 35 kDa (S.E. =  $\pm 3\%$ ) (Fig. 6C). To confirm that HER3-ECD<sup>I-II</sup> is a monomer, we used ultracentrifugation at three different concentrations and three different speeds.

- 5 The estimated average molecular weight of HER3-ECD<sup>I-II</sup>, using a group analysis of all of the scans, is  $35,000 \pm 210$  daltons (Fig. 6D). This is consistent with the expected molecular mass (37.5 kDa) of monomeric HER3-ECD<sup>I-II</sup>.

We used two independent methods to demonstrate direct binding of hrg to HER3-ECD<sup>I-II</sup>: a pull-down assay and surface plasmon resonance (SPR; BIACore) analysis. Binding of hrg to HER3-ECD<sup>I-II</sup> was shown using a pull-down assay under physiological salt conditions. In this analysis, we measured the amount of the V5-tagged HER3-ECD<sup>I-II</sup> that dissociated from immobilized trx-hrg by the addition of hrg. Using the trx-hrg-coupled S-protein-agarose, HER3-ECD<sup>I-II</sup> could be eluted with 1  $\mu$ M hrg (Fig. 7A, lane 4). In contrast, neither PBS (Fig. 7A, lane 3) nor EGF (Fig. 7A, lane 5) was effective in eluting HER3-ECD<sup>I-II</sup>.

10 To further confirm that HER3-ECD<sup>I-II</sup> binds hrg, we analyzed the interaction by SPR (BIACore). Trx-hrg fusion protein was immobilized on a BIACore chip for these measurements. SPR measurements using HER3-ECD<sup>I-II</sup> showed binding to the immobilized trx-hrg with a calculated equilibrium dissociation constant of 68 nM, calculated directly from  $k_{on}$  and  $k_{off}$  ( $k_{on} = (1.07 \pm 0.1) \times 10^5$  M<sup>-1</sup> s<sup>-1</sup>;  $k_{off} = (7.27 \pm 1) \times 15$  10<sup>-3</sup> s<sup>-1</sup>) (Fig. 7b). This interaction could be inhibited by stoichiometric concentrations of hrg. Based on these results, we conclude that HER3-ECD<sup>I-II</sup> containing domains I and II of HER3 binds hrg.

Example 12: Identification, Generation and Characterization of HER3 Residues Involved in Heregulin Binding

A. *Generation of trx-hrg and HER3 Point Mutants*

5 A thioredoxin-heregulin fusion (trx-hrg) was generated and purified as described elsewhere (see, e.g. Landgraf et al. (1998) *Biochemistry* 37(9), 3220-8). The HER3 point mutants were generated using the method of oligonucleotide directed mutagenesis described by Kunkel (see, e.g. Kunkel, T. A. (1988) *Nucleic Acids Symp Ser* (19), 43). Table 1 contains the oligonucleotides used for the mutagenesis. The PCR fragments were  
10 subcloned into the *Bgl* 2 and EcoR-1 site of HER3<sup>I-II</sup> (residues 20-329) and HER3<sup>I-IV</sup> (residues 20-643) in the pMT/BiP/V5-His-A expression vector (Invitrogen, Carlsbad, CA) and confirmed by sequencing. The mutants were expressed and purified as described elsewhere (see, e.g. Singer et al. (2001) *J Biol Chem* 276(47), 44266-74).

15 B. *Quantitation of the HER3 Point Mutants*

The concentration of the mutants were determined by analyzing the samples by SDS-PAGE on a 4-15% gradient gel (Biorad) followed by Western Blotting and chemiluminescence using a monoclonal antibody directed against the V-5 epitope conjugated to horse radish peroxidase (Invitrogen). Each mutant was run in triplicate  
20 and compared to a known concentration of HER3<sup>I-II</sup> (see, e.g. Singer et al. (2001) *J Biol Chem* 276(47), 44266-74).

C. *Binding Assays, Mass Spectrometry and CD Analysis:*

25 The gel shift assay, "pull down" assay, and surface plasmon resonance measurements were performed as described elsewhere (see, e.g. Singer et al. (2001) *J Biol Chem* 276(47), 44266-74). Matrix-assisted Laser Desorption Ionization (MALDI) time-of-flight mass spectrometry was performed as described elsewhere (see, e.g. Singer et al. (2001) *J Biol Chem* 276(47), 44266-74). The CD analysis was performed using a circular dichroism instrument from AVIV (Lakewood, NJ) with a 0.1 mM pathlength. The scan

was performed from 260nM to 195nM every 1.0 nM with 1.50 nM between scans and 3.0 seconds to collect each point.

D. *A mutation of residue 64 in HER3 to alanine abolishes heregulin binding*

5       HER3<sup>I-II E64A</sup> has a decreased affinity for heregulin binding. To locate the region of domain I that is critical for hrg binding, we expressed a series of point mutants in domain I of HER3<sup>I-II</sup> and assayed for hrg binding by two independent methods: a pull down assay and BIACore analysis. To determine whether the mutants of HER3<sup>I-II</sup> are capable of binding hrg, we used a "pull down" assay in which we measured the HER3<sup>I-II</sup>  
10      that could be dissociated from immobilized thioredoxin-heregulin (trx-hrg) by the addition of hrg. Using trx-hrg coupled S-protein agarose, HER3<sup>I-II</sup> was eluted with hrg and detergent (Figure 10A, lanes 3 and 5). No HER3<sup>I-II E64A</sup> could be recovered by either elution with hrg or detergent. (Figure 10A, lanes 4 and 6) This provides evidence that HER3<sup>I-II E64A</sup> does not bind hrg.

15       To further confirm that HER3<sup>I-II E64A</sup> does not bind hrg, we analyzed the interaction by surface plasmon resonance (SPR, BIACore). Trx-hrg fusion protein was immobilized on a BIACore chip for these measurements. We have previously shown that HER3<sup>I-II</sup> showed binding to immobilized trx-hrg with a calculated equilibrium dissociation constant of 68 nM using SPR measurements (see, e.g. Singer et al. (2001) *J Biol Chem* 276(47), 44266-74). HER3<sup>I-II E64A</sup> had a Kd of 95 nM, HER3<sup>I-II V110A</sup> had a Kd of 15 nM, (Table 2) HER3<sup>I-II E64A</sup> had no detectable binding above 500 nM to the immobilized hrg on the BIACore chip. (Figure 10B). All other mutants had a Kd similar to wild type. These results provide evidence that residues 44, 64, and 110 are in the heregulin binding region of HER3, and that residue 64 is critical for heregulin binding.

20       The residue at position 51 was also mutated to alanine because it could be protected by hrg from proteolytic cleavage, but was not directly in the binding footprint (Singer, 2001). HER3<sup>I-II K51A</sup> had the same Kd as HER3<sup>I-II</sup> (Table 2). This provides

evidence that residue 51 contributes no binding energy directly to HER3-heregulin interactions.

To show that the decreased binding affinity of HER3<sup>I-II</sup> E64A is not a result of proteolysis or misfolding, we performed mass spectrometry and CD analysis of HER3<sup>I-II</sup> E64A. The molecular mass of HER3<sup>I-II</sup> E64A is between 38.4 kDa and 39.8 kDa which is comparable to the molecular mass of HER3<sup>I-II</sup> (38.5 and 40 kDa) (Figure 11A). This provides evidence that the polypeptide chain of HER3<sup>I-II</sup> E64A is intact. The CD spectra of HER3<sup>I-II</sup> E64A and HER3<sup>I-II</sup> were essentially identical between 210nM and 260nM providing evidence that they have the same secondary structure. (Figure 11B). The fluctuations in the signal between 190nM and 210nM in HER3<sup>I-II</sup> E64A are probably due to aggregation. These results show that a mutation at residue 64 in HER3<sup>I-II</sup> has little affect on the structure of the protein, but results in decreased affinity for hrg.

To show that mutation at residue 64 has an effect in the extracellular domain of HER3, we expressed a series of mutants including HER3<sup>I-IV</sup> E64A and HER3<sup>I-IV</sup> and assayed for hrg binding by two independent methods: a native gel analysis, and a "pull down" assay. To determine the effects of cleavage of HER3<sup>I-IV</sup> on its ability to bind hrg, a gel mobility shift assay was performed. In a native gel analysis, HER3<sup>I-IV</sup> shows an electrophoretic mobility similar to HER3<sup>I-IVE64A</sup>. HER3<sup>I-IV</sup> shows a discrete shift in the presence of a 1:1 molar ratio of hrg to HER3<sup>I-IV</sup>. HER3<sup>I-IVE64A</sup> does not shift its gel position in the presence of hrg. (Figure 12A). This finding provides evidence that HER3<sup>I-IVE64A</sup> does not bind hrg.

To confirm that HER3<sup>I-IVE64A</sup> has decreased binding affinity for hrg, we used a "pull down" assay in which we measured the HER3<sup>I-IV</sup> and HER3<sup>I-IVE64A</sup> that could be dissociated from immobilized thioredoxin-heregulin (trx-hrg) by the addition of hrg. Using trx-hrg coupled S-protein agarose, HER3<sup>I-IV</sup> was eluted with hrg and detergent (Figure 12B, lanes 6 and 8), but not with PBS (Figure 12B, lane 4). No HER3<sup>I-IV</sup> E64A could be recovered by elution with PBS, hrg or detergent (Figure 12B, lanes 5, 7 and 9). This provided evidence that HER3<sup>I-IV</sup> E64A does not bind hrg.

E. *Amino Acids 64 and 110 are critical for heregulin binding*

In this study, we have identified two amino acids in discontinuous segments in domain I of HER3 which appear to be critical for heregulin binding. A mutation at residue 64 seemed to decrease all binding affinity in both recombinant H3 I-IV E64A and H3 I-II E64A. No detectable binding could be measured by gel shift, Biacore, or pull down assay. H3 I-II E64A seemed to be correctly folded as determined by CD analysis. These experimental results provide evidence that the decrease in binding affinity observed with this mutation is probably due to direct effects on interaction between H3 I-II E64A and heregulin rather than the consequences of misfolding or proteolysis of the mutant protein. A mutation at residue 110 (H3 I-II V110A) caused a 4 fold increase in binding affinity. A mutation at residue 44 (H3 I-II E44A) had a 1.5 fold decrease in binding affinity. A mutation at the protected proteolysis site (H3 I-II K51A) had no effect on binding affinity. These results provide evidence that amino acids 64 and 110 are critical for heregulin binding.

F. *Residues 64 and 110 map to the Hormone Binding Footprint in the IGF-1R Structure.*

Residues 64 and 110 are in domain I of HER3 and map to residues H30 and L81 in the hormone binding footprint in the IGF-1R (see, e.g. Garrett et al. (1998) *Nature* 394(6691), 395-9). (Figure 13). They are within 12Å of each other although they lie on different strands. They are within 22Å of the protected proteolysis site (see, e.g. Singer et al. (2001) *J Biol Chem* 276(47), 44266-74). Their close proximity to each other and the proteolysis site provide evidence that both residues may be involved in ligand binding.

G. *Residues 64 and 110 could form specific interactions with heregulin*

The five N-terminal residues of heregulin are required for specific interaction with HER3 (see, e.g. Jones et al. (1998) *J Biol Chem* 273(19), 11667-74). A valine lies at position 180 and a histidine is at position 178. H3 V110 and hrg V180 could form

hydrophobic interactions. H3 E64 and hrG H180 could form a salt bridge. (Figure 14). Mutation of these residues to alanine could disrupt these specific interactions.

Residue 50 can be protected by hrG from proteolysis but still not be involved directly with hrG binding. Our results provide evidence that residue 50 is not directly involved in hrG binding because a mutation at this site had no effect on hrG binding affinity. We propose that the N-terminus of hrG binds to residues 64 and 110 and that the omega loop blocks the protected proteolysis site (residue 50) by sterically hindering interaction with trypsin (Figure 15). This figure also shows how hrG in this orientation could fit into the cleft formed by domains I, II, and III as was proposed by (see, e.g. Garrett et al. (1998) *Nature* 394(6691), 395-9) and (see, e.g. Jorissen et al. (2000) *Protein Science* 9, 310-324) for the IGF-1R and IR.

We have identified two residues in HER3 that appear to be involved in hrG binding utilizing results from an alanine scanning mutagenesis of the related insulin receptor (see, e.g. Williams et al. (1995) *J Biol Chem* 270(7), 3012-6) and information from the hormone binding footprint of the known structure of the Insulin growth Factor Receptor (see, e.g. Garrett et al. (1998) *Nature* 394(6691), 395-9). A multiple sequence alignment of these three receptors allowed us to identify residues which were conserved among all three and were in or near the hormone binding footprint of the IGF-1R (see, e.g. Singer et al. (2001) *J Biol Chem* 276(47), 44266-74). This allowed us to choose which residues would have the highest probability of forming interactions with heregulin. We identified two residues that were critical for heregulin binding. We observed that complementary residues in heregulin that might form specific interactions with HER3. However, while this analysis implies direct molecular interactions between HER3 and heregulin, it is not direct proof. A high resolution structure of the HER3-heregulin complex is required to confirm these interactions.

**Tables****Table 1:** Oligonucleotide sequences used for generating the point mutants.

	<u>N-terminal primer</u>	<u>C-terminal primer</u>
HER3 I-II (residue 20)	CTA CTC TCT AGA TCC GAG GGC AAC TCT (SEQ ID NO: 9)	HER3 I-II (residue 329) TAC CGA TCT AGA TTT CGG ACA GAG ACC CCC (SEQ ID NO: 10)

<u>HER3</u> <u>Mutants</u>	<u>Plus Strand</u>	<u>Minus Strand</u>
E43A	GCGATGCTGCGAACCAATACC (SEQ ID NO: 11)	GGTATTGGITCGCAGCATCGC (SEQ ID NO: 12)
N44A	CGATGCTGAGGCCAACATACCAG (SEQ ID NO: 13)	CTGGTATTGGCGCTCAGCATCG (SEQ ID NO: 14)
K51A	GACACTGTACCGCCTCTACGAG (SEQ ID NO: 15)	CTCGTAGAGCCGGTACAGTGTC (SEQ ID NO: 16)
E64A	GGGAACCTTGCATTGTGCTC (SEQ ID NO: 17)	GAGCACAAATCGCAAGGTTCCC (SEQ ID NO: 18)
V66A	CTTGAGATTGCGCTCACGG (SEQ ID NO: 19)	CGTGAGCGCAATCTCAAGGT (SEQ ID NO: 20)
F94A	CCATGAATGAAGCCTCTACTCTAC (SEQ ID NO: 21)	GTAAGTAGAGCGTTCATTCATGG (SEQ ID NO: 22)
V110A	GACCCAGGCCTACGATGGGAA (SEQ ID NO: 23)	CTTCCCATCGTAGGCCTGGGTC (SEQ ID NO: 24)

5

**Table 2:** Dissociation constants for the binding of heregulin to alanine mutants of HER3 I-II. Recombinant HER3 alanine mutants were bound to immobilized hrg on the

BIACore chip. HER3 I-II showed binding to immobilized trx-hrg with a calculated equilibrium dissociation constant of 68 nM (see, e.g. Singer et al. (2001) *J Biol Chem* 276(47), 44266-74). The mutants are designated by the residue being mutated followed by the number indicating the position in the HER3 sequence, followed by alanine. Thus 5 V110A is a mutant in which valine at position 110 has been mutated to alanine. ND > 500nM indicates that no detectable binding was observed above 500nM. These results indicate that a mutation of valine at position 110 increased binding affinity 5 fold, while a mutation of the glutamate at position 64 nearly abolished binding affinity.

H3 I-II Mutant	Kd (nM)
E43A	63.0 +/- 6.4
N44A	95.5 +/- 15.6
K51A	63.0 +/- 6.4
E64A	ND > 500 nM
V66A	60.5 +/- 6.3
V110A	15.4 +/- 3.1

10

15

**Table 3:** Additional amino acid residues in HER3 associated with heregulin binding as determined by homology, crystallographic and solvent accessibility criteria.

	IGF-1R 2)	HER3 amino acid residues (SEQ ID NO: 2)
20	5-14	30-46
	27-29, 31-36	61-63, 65-70
	52-61	88-96
	77-79, 81-90	106-109, 111-120
	107-115	143-151
25	133-141	169-176

Residues include those in HER3 that lay on the face of the HER3 domain that includes the mutated residues characterized herein.

Table 4: Illustrative HER3, Hereregulin and HER2 polynucleotide and polypeptide sequences:

HER3 Polynucleotide Sequence

5 ATGAGGGCGAACGACGCTCTGCAGGTGCTGGGCTTGCTTTCAAGCCTGGCCCGGGCTCGAGGTGGC  
 AACTCTCAGGCAGTGTGCTGGACTCTGAATGCCCTGAGTGTGACCGGGATGCTGAGAACCATAAC  
 CAGACACTGTACAAGCTCACAGGAGGTGTGAGGTGGTATGGGAACTTGGAGATTGTGCTCACGGGA  
 CACAATGCCACCTCTCCCTCGAGTGGATTGAGAAGTGCAGGGCTATGCTCGTGGCCATGAAT  
 10 GAATTCTCTACTCTACCATGCCAACCTCCCGTGGTGGAGGGACCCAGGTACAGTGGAAAGTT  
 GCCATCTTCGTCATGTTGAACTATAACACCAACTCCAGGCCACGCTCTGCCAGCTCCGTTGACTCAG  
 CTCACCGAGATTCTGTCAGGGGGTGTATATTGAGAAGAACGATAAGCTTGTACATGGACACAATT  
 GACTGGAGGGACATCGTAGGGACCGAGATGCTGAGAGATGTTGAGAAGAACATGGCAGAAGCTGTCCC  
 CCCTGTCTGAGGTTGCAAGGGGGCATGCTGGGGCTCTGGATCAGAACAGACTGCCAGACATTGACCAAG  
 15 ACCATCTGTCCTCTCAGTGTAAATGGTCACTGCTTGGGCAAACCCAAACAGTGTGCTGCCATGATGAG  
 TGTGCGGGGGCTGTCAGGGACACAGACTGCTTGGGCACTTCATGACAGTGGAA  
 GCCTGTGTAACCTGCTGTCACAGCTCTTGTACAACAGTAACCTTCCAGCTGGAACCCAATCCC  
 CACACCAAGTATCAGTATGGAGGAGTTGTGAGGCCAGTGTCCCCATAACTTGTGGGGATCAAACA  
 TCCTGTGTCAGGGCTGTCCTGACAAGATGGAAGTAGATAAAAATGGGCTCAAGATGTGAGCCT  
 20 TGTGGGGACTATGTCCCAAAGCCTGTGAGGGAACAGGCTCTGGAGGCCCTCAGACTGTGGACTCG  
 AGCAACATTGATGGATTGTGAACTGCACCAAGATCTGGCAACCTGGACTTCTGATCACGGGCCTC  
 ATGGAGACACCTGGCAACAGATCTGGCCCTGGGACAGAGCTCAATGCTTCCGGACAGTACGG  
 GAGATCACAGGTTACCTGAAACATCCAGCTCTGGGCCACATGCACAACATTGCTTGTGGGGGGGGGG  
 25 TTGACAACATTGGAGGCGAGAACGCTCTACAACAGGGGCTTCTCATTGTTGATCATGAGAACATTGAAT  
 GTCACATCTGGGCTTCCGATCCCTGAAGGAAATTAGTGTGGGGCTATCTATATAAGTGCCTAG  
 CAGCTGCTACCACCACTCTTGAACCTGGACCAAGGTGTCTGGGGGCTACGGAAAGAGCGACTAGAC  
 ATCAAGCATATACTGGCGCGAGAGACTGCGTGGCAGAGGGCAAAGTGTGTACCCACTGTGCTCCT  
 GGGGGATGCTGGGGCCAGGGCCCTGGTCAGTGTCTGTGAAATTATAGCGGAGGGGTGTCTGT  
 GTGACCCACTGCAACTTCTGAATGGGAGCCTGGCTGAGAATTTCGGCCATGAGGCCAATGCTTCTCTGC  
 30 CACCGGAATGCAACCCATGGGGGCACTGGCACATGGCTGGGGCTCTGATACTTGTGCTCAA  
 TGTGCCATTGGAGATGGGGGCACTGTGTGAGCAGCTGCCCATGGAGTCTTAGGTGCAAGGGC  
 CCAATCTACAAGTACCCAGATGTTGAGAATGAATGTCGGCCCTGGCATGAGAACCTGCACCCAGGGTGT  
 AAAGGACAGAGCTCAAGACTGTTAGGACAAACACTGGTGTGATGGCAAAACCCATCTGACAATG  
 GCTTTGACAGTGATAGCAGGATTGGTAGTGTGATTTCATGATGTCGGGGCACTTTCTACTGGCGT  
 35 GGGCGCGGATTAGAATAAAGGGCTATGAGGGCAACTTGGAAAGGGGGTGGAGAGCATAGAGCCTCTG  
 GACCCAGTGAAGGCTAACAAAGTCTGGCAAGAATCTTCAACAGAGACAGAGCTAACAGGCTTAA  
 GTGCTGGCTGGGTCTTGGAAACTGTGCAACAAAGGAGTGTGGATCCCTGGGGGTGAATCAATCAAG  
 ATTCCAGTCTGCATTAAAGTCATTGAGGACAAGAGTGGACGGCAAGAGTTCAAGCTGTGACAGATCAT  
 ATGCTGGCATTGGCAGCTGGACCATGCCACATTGTAAGGTGCTGGACTATGCCAGGGCTACT  
 40 CTGCAGCTGCACTCAATATTGCTCTGGGCTCTGCTGATCATGTGAGACAACACGGGGGCA  
 CTGGGGCCACAGCTGCTCAACTGGGAGTACAAATTGCCAACGGGAATGTACTACCTTGAGGAACAT  
 GGTATGGTGATAGAACCTGGCTGCCAACGTGCTACTCAAGTCACCCAGTCAGGTGCTAGGTGGCA  
 GATTTGGTGTGGCTGACTCTGCTGCCCTCTGATGATAAGCAGCTGCTACAGTGAGGCCAAGACTCCA  
 ATTAAGTGGATGGCCCTTGAGAGTATCCACTTGGGAAACACACACAGAGTGTGAGTGTGGAGCTAT  
 45 GGTGTGACAGTTGGAGTTGATGACCTCAGGGGCAAGGCCATGCAAGGGCTACGATTGGCTGAAGTA  
 CCAGACCTGCTAGAGAAGGGGGAGCGGTTGGCACAGGCCAGATCTGCCACATTGATGCTACATGGTG  
 ATGGTCAGTGTGGATGATTGATGAGAACATTGCCAACCTTAAAGAACACTAGGCCATGAGTTGCC  
 AGGATGGGGGAGACCCACCACGGTATCTGGTCATAAAAGAGAGAGTGGGGCTGGAAATGCCCTGG  
 CCAGAGCCCCATGGTGTGACAACAAAGAAGCTAGAGGAAGTAGAGCTGGAGGCCAGACTAGACCTAGAC  
 50 CTAGACTTGGAAAGCAGAGGAGGACAACCTGGCAACCACCAACTGGGCTCCGCCCTCAGCCTACCAAGT  
 GGAACACTTAATCGGCCACGTGGGAGGCCAGGCCCTTTAAGTCCATCATCTGGATACTGCCATGAAC  
 CAGGGTAATCTGGGGGCTTGCCAGGAGTCTGAGTTCTGGGAGCAGTGAACGGTGGCCCCGTCCA  
 GTCTCTACACCCAATGCCACGGGGATGCCATGAGTCAGTCAAGAGGGCATGTAACAGGCTCT

GAGGCTGAGCTCCAGGAGAAAGTGTCAATGTGAGAAGCCGGAGCAGGAGCCACGCCACGG  
 GGAGATAGCGCTACCATCCCAGGCCACAGTCTGCTACTCTGTTACCCACTCTCCCCACCCGG  
 TTAGAGGAAGAGGATGTCAACGGTTATGTCAATGCCAGATAACACACCTCAAAGGTA  
 CTCCCTCTCCCTGGGAGAAGAAGATGAAGAGATGAAGATGAGGAG  
 5 TATGAATACATGAACCGGAGGAGAAGGCACAGTCACCTCATCCCCCTAGGCCAAGTTCCCTTGAGGAG  
 CTGGTTATGAGTACATGGATGTGGGTCAGACCTCAGTCCTCTGGCAGCACACAGAGTTGCCA  
 CTCCACCCCTGTACCCATCATGCCACTGCAGGACAACACTCCAGATGAAGACTATGAATATGAATCGG  
 CAACGAGATGGAGGTTCTGGGGGTGATTATGCAGCCATGGGGGCTGCCAGCATCTGAGCAAGGG  
 TATGAAGAGATGAGAGCTTCAGGGCCTGGACATCAGGCCCCCATGTCATTATGCCCTAA  
 10 ACTCTACGTAGCTTAGAGGCTACAGACTCTGCCATTGATAACCTGATTACTGGCATAGCAGGTTTC  
 CCCAAGGCTAATGCCAGAGAACG SEQ ID NO: 1

## 15 HER3 Polypeptide Sequence

MRANDALQVLGLLFLS LARGSEVGN SQAVCPGT LNGLSVT GDAENQY QTL YKLY ERCEVMGN LEIVLTG  
 HNA DLS FLOWIREVTGYVLVAMNEFSTLPLPNLRVRGTOVYDGKFAIFVMLNYNTNSHALRQLRLTQ  
 20 LTE ILSGGV YIEKNDKLCHMDTIDWRD IDIVRDRDAEIVVKDNGRSCPPCHEVKGR CWGPG SEDCQTLTK  
 TICAPQCNGHCFGPNPNCQCHDECAGCGSPQDTCFACRHFNDSGACVPRCPQPLVYNKLT FQLEPNP  
 HTKYQYGGVCVASCPHN FVVDQTS C VRACPPDKM EVDKNGLK MCEPCGGLCPKACE GTGS GS RFTQ VDS  
 SNIDGFVNCTKILGNLD FLITGLNGDPW KIPALDPEKLN VFRTVREITGYLN IQSWPPHMHNFSVFSN  
 LT TIGGRSL NRG FSL IMK NLN VTS LGFR S LKE ISAGRI YI SANRQLCYHSLNWTKVLRGPTEERLD  
 25 IKHNR PRRDCVAEGK VCDPLCS SGCGWGP GQCL SCR NYSRGCVTHCNFLNGEPREF AHEAE C FSC  
 HPECQPMGGTATCNGS D TCAQCAHFRDGP HCVSSCPHVG LAKGPI YKYPDVQNECRP CHENCTQGC  
 KGPELQDCLGQ TLV LIGK THLT MALT VIAGL VV IFMMLGGTFLYW RGRRI QNK RAM RRY LERGES IEPL  
 DPSEKAN KV LARIFKETELRK LKVL GSGVFGT VHKGV WIP EGESIKIPVCIKVIEDKS GRQS FQAVTDH  
 MLAIGSLDHAHIVRLLGLCPGSSQLQVLTQYLP LGSLLDHVRQH RGA LGPQ LLLNWGVQIAKG MYL EEH  
 GMVHRNL AARNVLLKSPSQVQVADFGVADLPPDKQ LLYSEAKT PI KWM ALESIHFGKYTHQSDVWSY  
 30 GVTWELMTFGAEPYAGLRLAEPV DLEKGERLAQPQIC TIDV YMVMV KCW MIDE NIRPTF KELAN EFT  
 RMARD PPRYL V KRES GP GIA PGPEPH GLTNK LLEVEPEL DLD LEEEDN LATT TLG SAL SLPV  
 GTLNR PRGS QSL LPSG SGYMPM NQGNLGGSC QES AVSG SSER C PRPV SLH PMPRG CLAS ESEG HV TGS  
 EAELQE KVSMCR SRS RSPR PRGDSAYHSQRH SLLTPV TPLS PPGLE EEDV NGYVMPD THLK GTPSSR  
 35 EG TLSSV GLS VLG TEE EDE DEE Y EY MNRRRRHSPH PPRPSS LEE LGY EY MDVG SDLS ASLG ST QSCP  
 LHPV PIMPTAGTT PDED EY EMNR QRDGGGP GGDY AAMGACPASEQGY EEMRAF QGP GHQ APHV YARLK  
 T LRSLE ATDSA FDNP DPDY WHS RLFP KANA QRT SEQ ID NO: 2

## 40 Heredulin Polynucleotide Sequence

ATGTCCGAGCGCAAAGAAGGCAGAGGCAAAGGGCAAGAAGAAGGAGCGAGGCTCGGCAAGAAG  
 CCCGAGTCCCGGGCGGGCAGCCAGAGCCCAGCCTCTCCCAATTGAAAGAGATGAAAAGCCAGGAA  
 TCGGCTGCAGGTTCAAAC TAGTCCTCGGTGTGAA ACCAGTTCTGAATACTCCTCTCAGATTCAAG  
 TGGTTCAAGAATGGGAATGAATTGAATCGAAAAACAAACCACAAAATATCAAGATACAAAAAAAGCA  
 45 GGGAGTCAGAAC TCGCATTAACAAAGCATCACTGGCTGATTCTGGAGAGTATATGTGCAAAGTGATC  
 AGCAAATTAGGAAATGACAGTGCCTCTGCCAATATCACC ATCGTGGAAATCAAACGAGATCATCACTGGT  
 ATGCCAGCCTCAACTGAAGGAGCATATGTGCTTCAGAGTCTCCATTAGAATATCAGTATCCACAGAA  
 GGAGCAAATACTCTCTCATCACATCTACATCCACACTGGGACAAGCCATCTGTAAAATGTGCGGAG  
 AAGGAGAAAAC TTTCTGTGTGAATGGAGGGAGTGTCTCATGGT GAAAGACCTTCAACCCCTCGAGA  
 50 TACTTGTGCAAGTGC CCAAATGAGTTACTGGT GATCGCTGCCAAA ACTACGTAATGGCCAGCTTCTAC  
 AACGATCTTGGGATTGAATT TATGGAGGGCGAGGAGCTGTAC CAGAAGAGAGTGTGCTGACCATAACCGG  
 ATCTGCATGCCCTCTGTGGT CGGCATCATGTGTG TGCTACTGC AAAACCAAGAAACAGCGG

AAAAAGCTGCATGACCGTCTTCGGCAGAGCCTCGGTCTGAACGAAACAATATGATGAACATTGCCAAT  
 GGGCCTCACCATCCTAACCCACCCCCGAGAATGTCCAGCTGGTGAATCAATACGTATCTAAAACGTC  
 ATCTCCAGTGAGCATATTGAGAGAGAAGCAGAGACATCCTTTCCACCAACTCACTATACTCCACA  
 GCCCATCACTCCACTACTGTACCCAGACTCTAGCCACAGCTGGAGCAACGGACACACTGAAAGCATC  
 5 CTTTCCGAAAGCCACTCTGTAATCGTATGTCATCCGTAGAAAACAGTAGGCACAGCAGGCCAAGTGG  
 GGCCCAAGAGGAGCTTAAATGCCACAGGAGGCCCTCGTGAATGTAACAGCTTCCTCAGGCATGCCAGA  
 GAAACCCCTGATTCTAACCGAGACTCTCCTCATAGTGAAGGTATGTGTCAGCCATGACCAACCCGGCT  
 CGTATGTCACCTGTAGATTTACAGCAGCTCCCCAAATCGCCCCCTCGGAAATGTCCTCCACCC  
 GTGTCCAGCATGACGGTGTCCATGCCATGGCGGTAGCCCCCTCATGGAAGAAGAGAGACCTCTA  
 10 CTTCTCGTGAACACCACCAAGGCTGCCAGAGAAGAAGTTGACCATCACCCCTAGCAGTTCAAGCTCC  
 CACACAAACCCCGCGCATGACAGTAACAGCCTCCCTGCTAGCCCCCTGAGGATAGTGGAGGATGAGGAG  
 TATGAAACGACCCAAGAGTACGAGGCCAGGCCAAGAGCCTGTTAAGAAACTGCCAATAGCCGGGGCC  
 AAAAGAACCAAGCCAAATGCCACATTGTAACAGATTGGAAGTGGACAGAACACAAGCTCCCAGAGC  
 AGTAACACTAGAGAGTGAAACAGAAAGATGAAAGAGTAGGTGAAGAATACGCCCTTCTGGCATAACAGAAC  
 15 CCCCTGGCAGCCAGTCTGAGGCAACACCTGCCCTGGCTGACAGCAGGACTAACCCAGCAGGC  
 CGCTCTCGACACAGGAAGAAATCCAGGCCAGGCTGTAGTGAATTGCTAACCAAGACCCATTGCT  
 GTA SEQ ID NO: 3

## 20 Heregulin Polypeptide Sequence

MSERKEGRGKGKKERGSKPESQSPALPPRLKEMKSQESAGSKLVLRCETSSEYSSLRFK  
 WFKNNGNELNRKPKPNQNIQKPKGKSELINKASLADSGEYMKVSKLGNDSASANITIVESNEIITG  
 MPASTEGAYVSSESPIRISVSTEGANSSSTSTTGTSVLVKCAEKEKTFCVNGECFMVKDLNSNPSR  
 25 YLCCKCPNEFTGDRCQNYVMASFYKHLGIEFMEAEELYQKRVLTITGICIAVVGIMCVVAYCKTKQR  
 KKLHDRLRQLSRLSERNNMNTANGPHPPNPPPENVQLVNQYVSKNVISSEHVEREAETSFSTSHYTST  
 AHHSTTVTQTPSHWSWNIGHTESILSESHSVIVMSVENSRHSSPTGGPRGRNLNGTGGPRECNFLRHAR  
 ETPDSYRDSPHSERYVSAMTPPARMSPVDFHTPSPKSPSEMSPVSSMTVSMPMSAVSPMEEERPL  
 30 LLVTPPRRLREKKFDHHPQQFSSFHNPADSNSLPASPLRIVEDEEYETTQEYEPAQEPVKLANSRRA  
 KRTKPNGHIANRLEVDNTSSQSSNSESETEDERVGEDTPFLGIQNPLAASLEATPAFLADSRTNPAG  
 RFSTQEEIQARLSSVIANQDPIAV SEQ ID NO: 4

## 35 HER2 Polynucleotide Sequence

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 CGCCACCTTACCAAGGGCTGCCAGGTGGCAGGGAAACCTGGAACCTCACCTACCTGCCACCAATGCC  
 40 AGCCTGTCTTCTGCAAGGATATCCAGGAGGTGCAAGGGCTACGTGCTCATCGCTCACACCAAGTGG  
 CAGGTCCCAC TGCAAGAGGCTGCCAGGATGTGCGAGGCACCCAGCTTTGAGGACAACATGCCCTGGCC  
 GTGCTAGACAATGGAGACCCGCTGAAACAAATACCAACCCCTGTACAGGGCTCAGGGCTCAGGG  
 GAGCTGCAGCTTCGAAGCCTCACAGAGATCTGAAAGGAGGGCTTGTACAGGGCTCAGGG  
 TGCTACCAAGGACACGATTGTGGAAGGACATCTCCACAAAGAACAAACAGCTGGCTCTCACACTGATA  
 45 GACACCAACCGCTCTGGCCTGCCACCCCTGTTCTCGATGTGTAAGGGCTCCGCTGCTGGGAGAG  
 AGTTCTGAGGATTGTCAAGCTGACGCCACTGTCTGTGCCGGTGGCTGTGCCGCTGCAAGGGCCA  
 CTGCCCACTGACTGCTGCCATGAGCAGTGTGCTGCCGGCTGACGGGCCCCAAGCACTGACTGCC  
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 50 CCCTACAACTACCTTCTACGGACGTGGGATCTGCAACCCCTGCTGTGCCGGTGTGCAACCAAGAGGTG  
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 AATGGCGCTACTCGCTGACCCCTGCAAGGGCTGGCATCAGCTGGCTGGGCTCGCCTACTGAGGGAA  
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 5 CAGCTTTGCGAACCCGACCAAGCTCTGCTCCACACTGCCAACCGCCAGAGGACGAGTGTGTGCG  
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 GTGG  
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 GCAGAGAACCCAGAGTACCTGGGTCTGGACGTGCCAGTG SEQ ID NO: 5

40. HER2 Polypeptide Sequence

MELAALCRWGLLLALLPPGAESTQVCTGTDMLRHLYQGCQVVQGNLELTYLPTNA  
 SLSFLQDIQEVEQGYVLIAHNQVRQVPLQRRLIVRGTQLFEDNYALAVLDNGDPLNNNTPVTGASPGGLR  
 45 ELQLRSLTEILKGGVLIQRNPQLCYQDTILWKDIFHKNNQLALTLIDTNRSRACHPCSPMCKGSRCWGE  
 SSEDCQSLTRTVCAAGGCARCKGPLPTDCCHEQCAAGCTGPKHSDCLACLHFNHSGICELHCPALVTNT  
 DTFSMPNPECGRYTFGASCVTPCYNLSTDVGSTLVCPLHNQEVTAEDGQTQCEKCSKPCARVCYGL  
 GMEHLREVRAVTSANIQEFAGCKKIFGLAFLPESFDGDPASMTAPLQPEQLQVFETLEEITGYLYISA  
 WPDSLPLSVFQNLQVIRGRILHNGAYSLTLQGLGISWLGLRSRRELGSGLALIHHNTHLCFVHTVPWD  
 50 QLFRRPHQALLHTANRPEDECVGEGLACHQLCARGHCWGPGBTQCVNCNSQFLRGQECVVECRVLQGLPR  
 EYVNARHCLPCHPECQPQNGSVTCFGPEADQCVACAHYKDPPFCVARCPGKPDLSYMPIWKFPDEEG  
 ACQPCPINCHSCVDLDDKGCPAEQRASPLTSIVSAVVGILLVVVLGVVFGILIKRQQKIRKYTMRL  
 LQETELVEPLTPSGAMPNQAQMRLIKETELRKVKVLGSGAFGTGYKGWIWPDGENVKIPVAIKVLR  
 SPKANKEILDEAYVMAGVSPYVSRLLGICLTSTVQLVTQLMYGCLLDHVRENRGRLGSQDLLNWCMQ

IAKGMSYLEDVRLVHRDLAARNVLVKSPNVKITDFGLARLLDIDETEYHADGGKVPIKWMALESILRR  
RFTHQSDVWSYGVTVWELMTFGAKPYDGIPAREIPDLLEKGERLPQPPICTIDVYMIMVKCWMIDSECR  
PRFRELVSEFSRMARDPQRFFVVIQNEDLGPASPLDSTFYRSLLIEDDDMGDLVDAAEYLVPQQGFFCPDP  
APGAGGMVHHRHRSSTRSGGDLTLGLEPSEEAPRSPLAPSEGAGSDVFDGDLGMGAAKGLQSLPTH  
5 DPSPLQRYSEDPTVPLPSETDGYVAPLTCSHQPEYVNQPDVRPQPPSPREGPLPAARPAGATLERAKTL  
SPGKNGVVKDVFAGGAVENPEYLTPQGGAAPQPHPPPSPAFDNLYWDQDPPERGAPPSTFKGTPT  
AENPEYLGLDVPV SEQ ID NO: 6

## WHAT IS CLAIMED IS:

1. A non-naturally occurring HER3 variant polypeptide consisting essentially of amino acid 20 to amino acid 329 of HER3 polypeptide sequence of SEQ ID NO: 2; wherein the HER3 variant polypeptide:
  - (a) specifically binds to the heregulin polypeptide of SEQ ID NO: 4; and
  - (b) exhibits an impaired ability to interact with the HER2 polypeptide of SEQ ID NO: 6.
2. A non-naturally occurring HER3 variant polypeptide consisting essentially of amino acid 19 to amino acid 329 of HER3 polypeptide sequence of SEQ ID NO: 2; wherein the HER3 variant polypeptide has the ability to inhibit the interaction between wild type HER3 having the polypeptide sequence of SEQ ID NO: 2 and heregulin having the polypeptide sequence of SEQ ID NO: 4.
3. The HER3 variant polypeptide of claim 1 further comprising one or more amino acid substitutions at the following residue position(s) in SEQ ID NO: 2: E43; N44; K51; E64; V66; and V110.
4. A HER3 variant polypeptide comprising an amino acid sequence which differs from the native sequence HER3 polypeptide sequence of SEQ ID NO: 2 and has one or more of amino acid substitutions at the following residue position(s) in SEQ ID NO: 2: E43; N44; K51; E64; V66; and V110.
5. An isolated nucleic acid comprising a nucleotide sequence encoding the HER3 variant of claim 4.
6. A vector comprising the nucleic acid of claim 5.
7. A host cell comprising the vector of claim 6.
- 25 8. The host cell of claim 7 wherein said host cell is *E. coli*.
9. A method of making HER3 variant polypeptide, comprising the steps of: providing a host cell comprising the vector of claim 6; (b) providing culture media; (c) culturing the host cell in the culture media under conditions sufficient to express the

HER3 variant polypeptide; (d) recovering the HER3 variant polypeptide from the host cell or culture media; and (e) purifying the HER3 variant polypeptide.

10. The HER3 variant polypeptide of claim 4, wherein the HER3 variant polypeptide is conjugated or linked to one or more polyol groups.

5 11. The HER3 variant polypeptide of claim 4, wherein the HER3 variant polypeptide has a substitution at V110.

12. The HER3 variant polypeptide of claim 11, wherein the HER3 variant polypeptide has an alanine substitution at V110.

13. The HER3 variant polypeptide of claim 4, wherein the HER3 variant 10 polypeptide has a substitution at E64.

14. The HER3 variant polypeptide of claim 4, wherein the HER3 variant polypeptide comprises a soluble, extracellular domain HER3 polypeptide.

15. The isolated HER3 variant polypeptide of claim 1, wherein the HER3 variant polypeptide does not have any portion of the amino acid sequence consisting of 15 amino acid 330 to amino acid 1342 of HER3 polypeptide sequence of SEQ ID NO: 2.

16. A method of identifying a compound which specifically binds the heregulin binding domain in a HER3 variant polypeptide comprising amino acid 20 to amino acid 329 of HER3 polypeptide sequence of SEQ ID NO: 2 comprising contacting the HER3 variant polypeptide with a test compound under conditions favorable to 20 binding and determining whether the test compound specifically binds to the HER3 variant polypeptide such that a compound which binds to the HER3 variant polypeptide can be identified.

17. The method of Claim 16, further comprising determining whether the test compound inhibits the heregulin induced tyrosine kinase activity associated with a 25 HER3 polypeptide having the sequence shown in SEQ ID NO: 2 comprising the steps of:

contacting mammalian cells that express HER3 receptor with heregulin in the presence and absence of the test compound; and

monitoring the mammalian cells for the tyrosine kinase activity associated with the HER3 polypeptide, wherein an inhibition in tyrosine kinase activity in the presence of the test compound as compared to the absence of the test compound identifies the test compound as an inhibitor of heregulin induced tyrosine kinase activity.

5 18. The method of Claim 16, further comprising determining whether the test compound enhances the heregulin induced tyrosine kinase activity associated with a HER3 polypeptide having the sequence shown in SEQ ID NO: 2 comprising the steps of:

10 contacting mammalian cells that express HER3 receptor with heregulin in the presence and absence of the test compound; and

monitoring the mammalian cells for the tyrosine kinase activity of the HER3 polypeptide, wherein an increase in tyrosine kinase activity in the presence of the test compound as compared to the absence of the test compound identifies the test compound as an enhancer of heregulin induced tyrosine kinase activity.

15 19. A method of determining whether a test compound modulates the interaction between heregulin polypeptide of SEQ ID NO: 4 and a HER3 variant polypeptide comprising amino acid 20 to amino acid 329 of HER3 polypeptide sequence of SEQ ID NO:2 comprising the steps of:

20 (a) contacting the HER3 variant polypeptide with a test compound under conditions favorable to binding;

(b) contacting the HER3 variant polypeptide of step (a) with heregulin under conditions favorable to binding;

25 (c) comparing the binding interaction between the HER3 variant polypeptide and heregulin of step (b) with the binding interaction between HER3 variant polypeptide and heregulin in the absence of the test compound such that a compound which modulates the interaction between heregulin and the HER3 variant polypeptide can be identified.

20. A method of inhibiting the interaction between a heregulin polypeptide having the sequence shown in SEQ ID NO: 4 and HER3 polypeptide having the

sequence shown in SEQ ID NO: 2 comprising exposing the heregulin polypeptide to a non-naturally occurring HER3 variant polypeptide comprising amino acid 20 to amino acid 329 of HER3 polypeptide sequence of SEQ ID NO: 2; wherein the HER3 variant polypeptide specifically binds to the heregulin polypeptide of SEQ ID NO: 4; and  
5 exhibits an impaired ability to interact with the HER2 polypeptide of SEQ ID NO: 6.

21. The method of claim 20, wherein the HER3 variant polypeptide has one or more of amino acid substitutions at the following residue position(s) in SEQ ID NO: 2: E64 and V110.
22. The method of claim 20, wherein the HER3 variant polypeptide has an  
10 amino acid substitution at V110.
23. The method of claim 20, wherein the amino acid substitution is V91A.
24. The method of claim 20, wherein the HER3 variant polypeptide is conjugated or linked to one or more polyol groups.
25. The method of claim 24, wherein the polyol group is polyethylene glycol.

15

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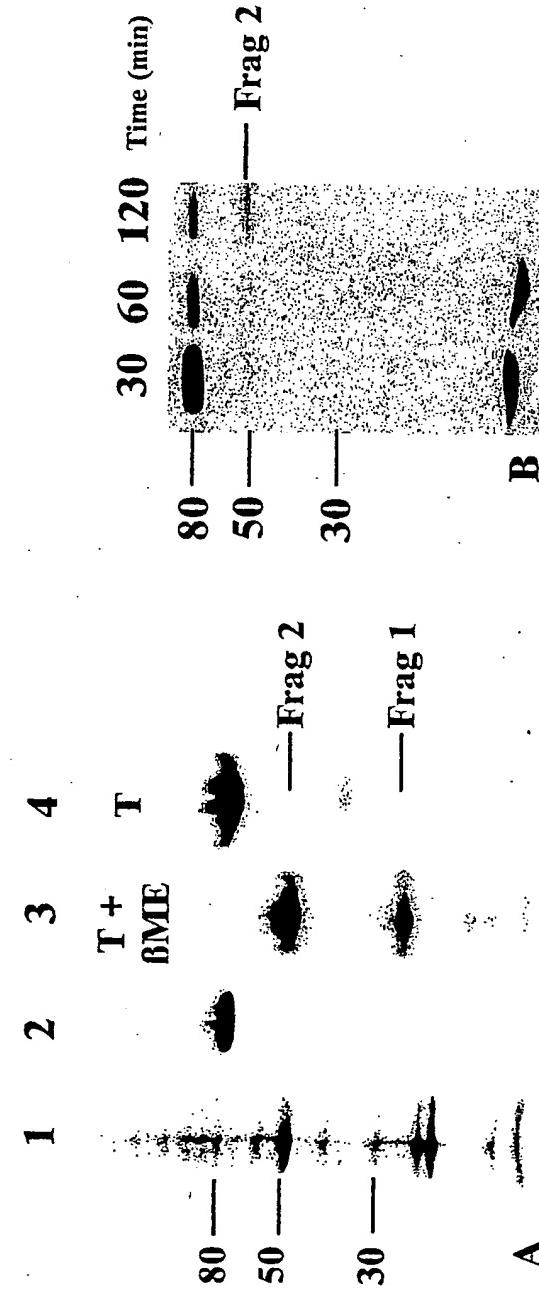


FIG. 1

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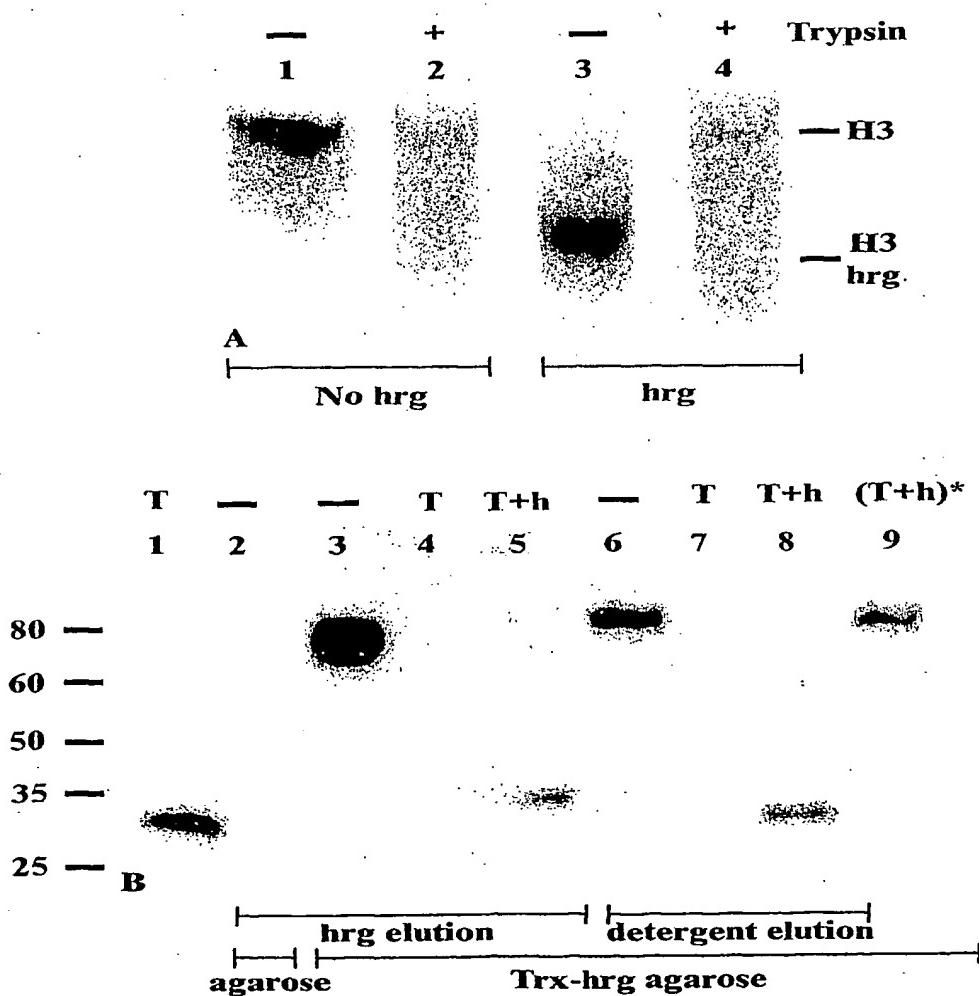


FIG. 2

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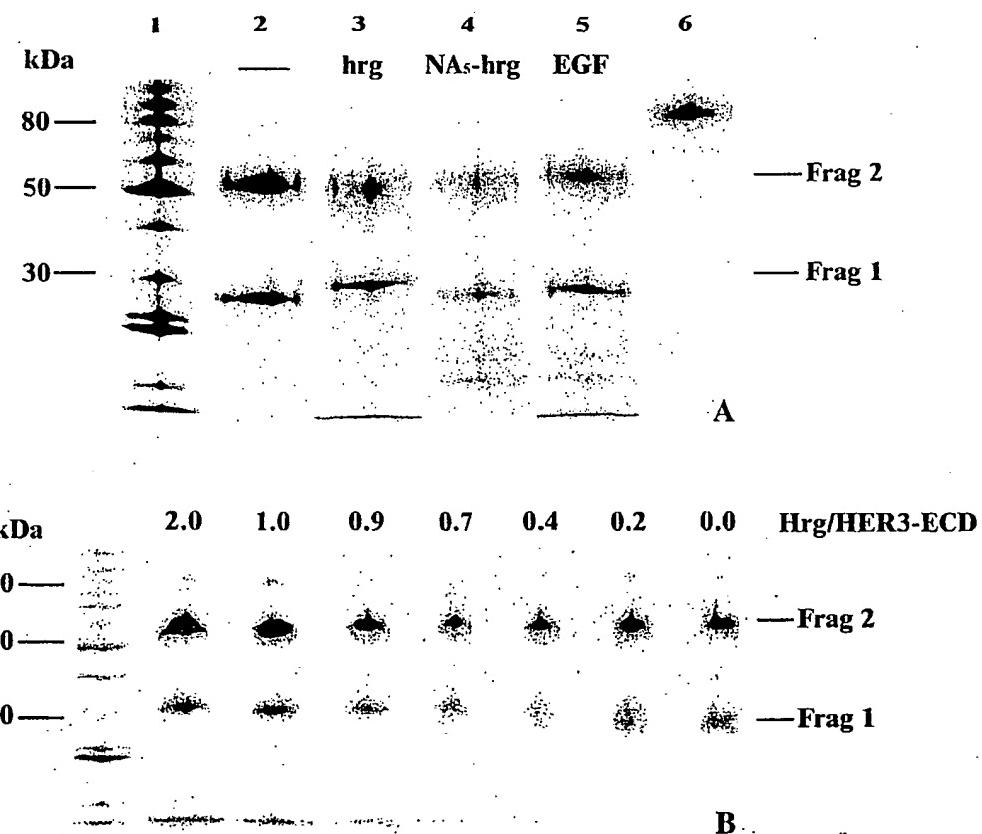


FIG. 3

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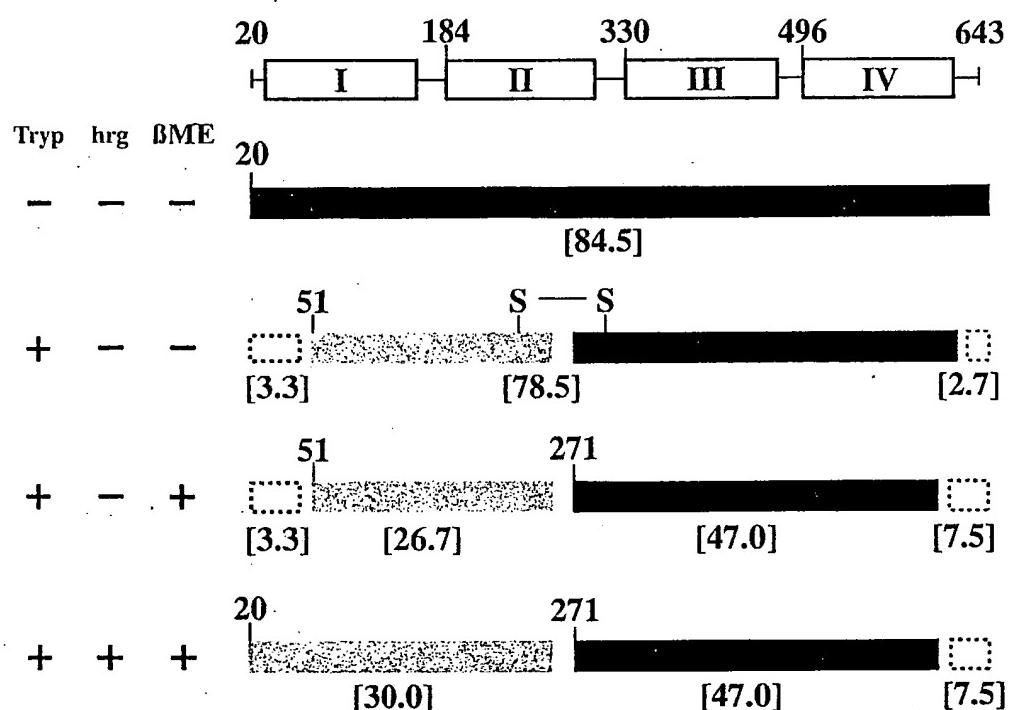
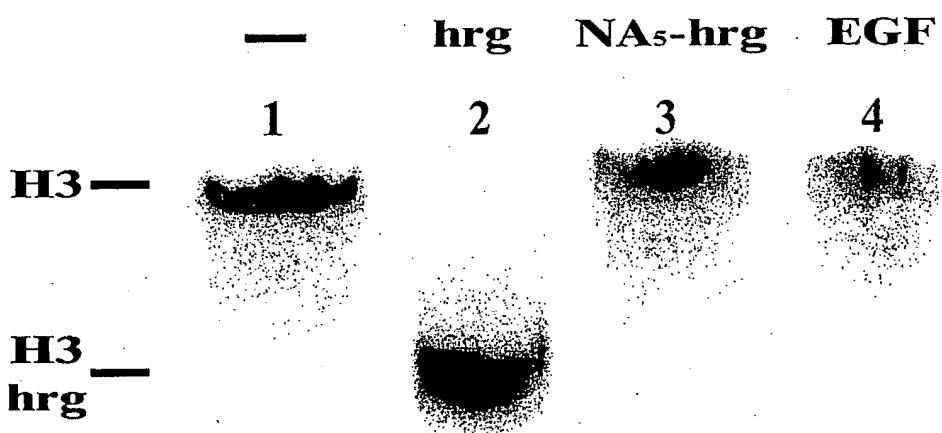


FIG. 4

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**FIG. 5**

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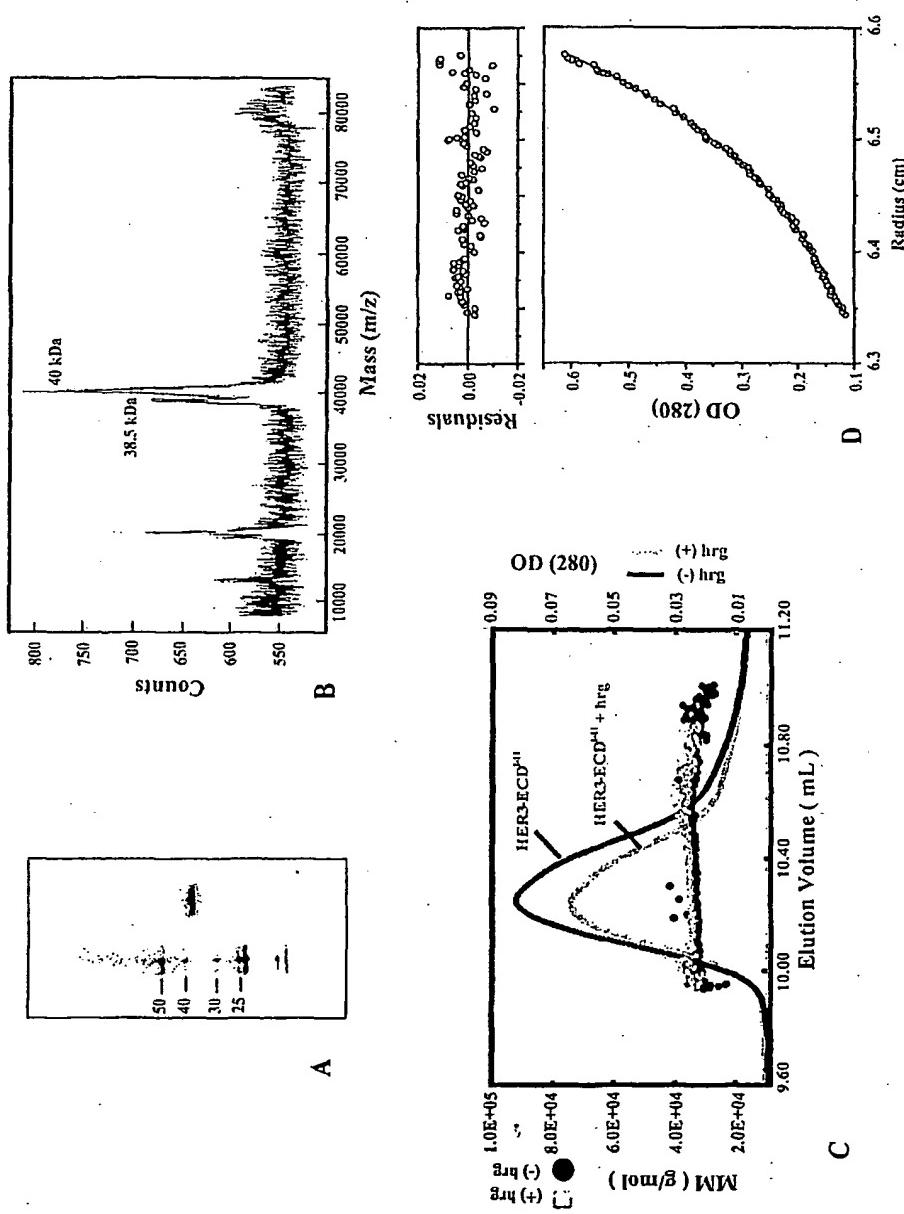


FIG. 6

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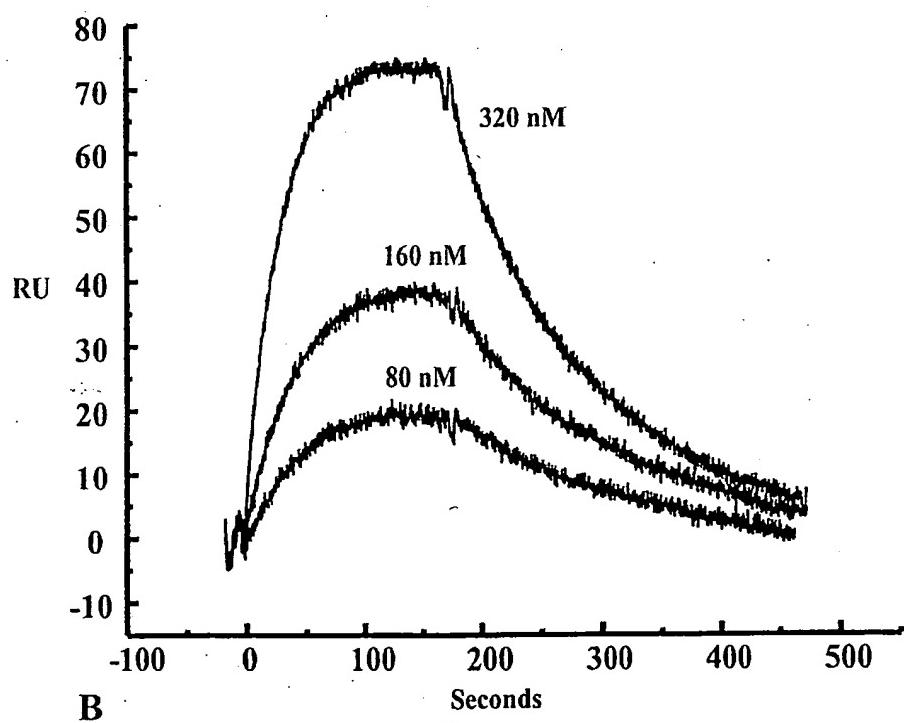
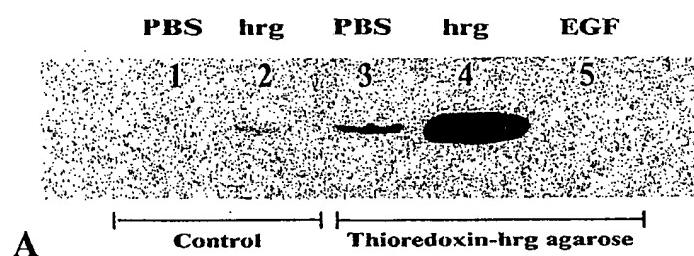


FIG. 7

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L1 1 - HLY PGEV C P G . M . . . .  
 L1 1 - - - - E I C G P G . I . . . .  
 P1R L1 20 SEVGN S Q A V C . P G T L N G L S V T  
 x3 1

\* \* \*

E G H L Q I L M F	K T R P E D F R D L	S F P K L I M I T D
E G Y L H I L L I S	K A . E D Y R S Y	R F P K L T V I T E
M G N L E I V L T G	H N A D L S E F L Q W	I R E V T G Y V L V

\* \* \*

E S L K D L E P P N L	T V I R G S R L F F	N Y A L V I F E M V
E S L G D L E P P N L	T V I R G W K L F Y	N Y A L V I F E M T
P N L R V Y R G T Q	V Y D G K F A I E V	M L N Y N T N S S H

O \*

E K N N E I L C Y L A	T I D W S R I I D S	V E D N H I M L N K
E K N A D I L C Y L S	T V D W S L I I D A	V S N N Y I V G N K
E K N D K L C H M D	T I D W R D I V R D	R D A E I V V . K

\* \* \*

M N I T R G S V R I	T I D W S R I I D S	V E D N H I M L N K
R N I T R G A I R I	T V D W S L I I D A	V S N N Y I V G N K
T E I I L S G G V I	T I D W R D I V R D	R D A E I V V . K

\* \* \*

D D N E E C G D		
P P . K E C G D		
D N G R S C P -		

FIG. 8

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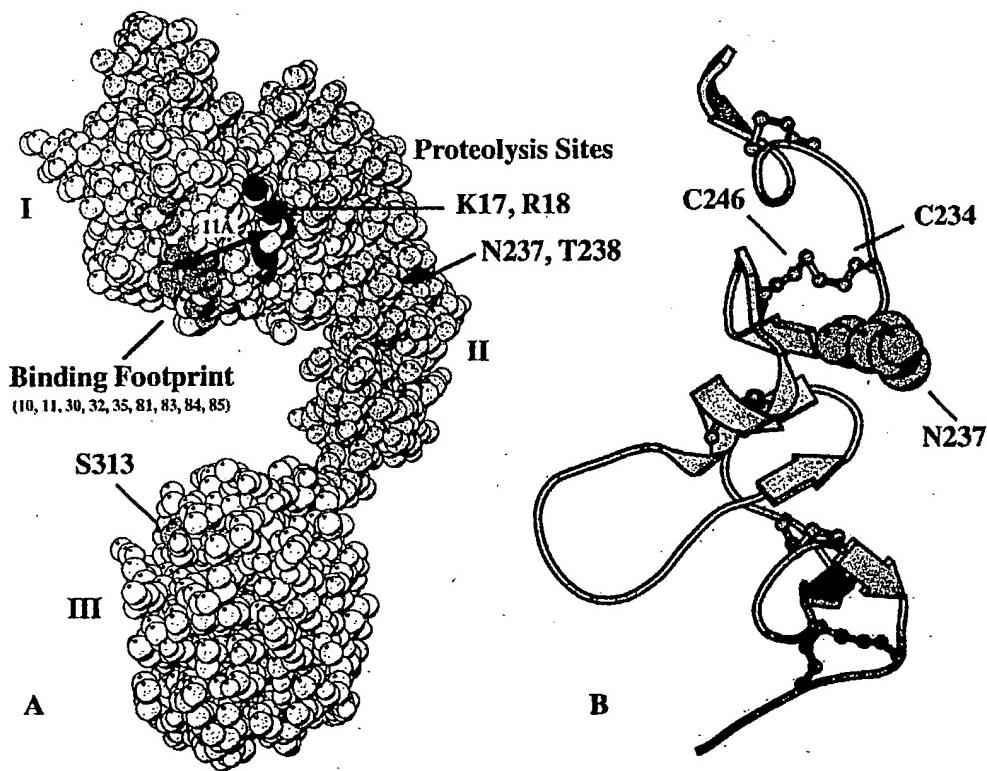


FIG. 9

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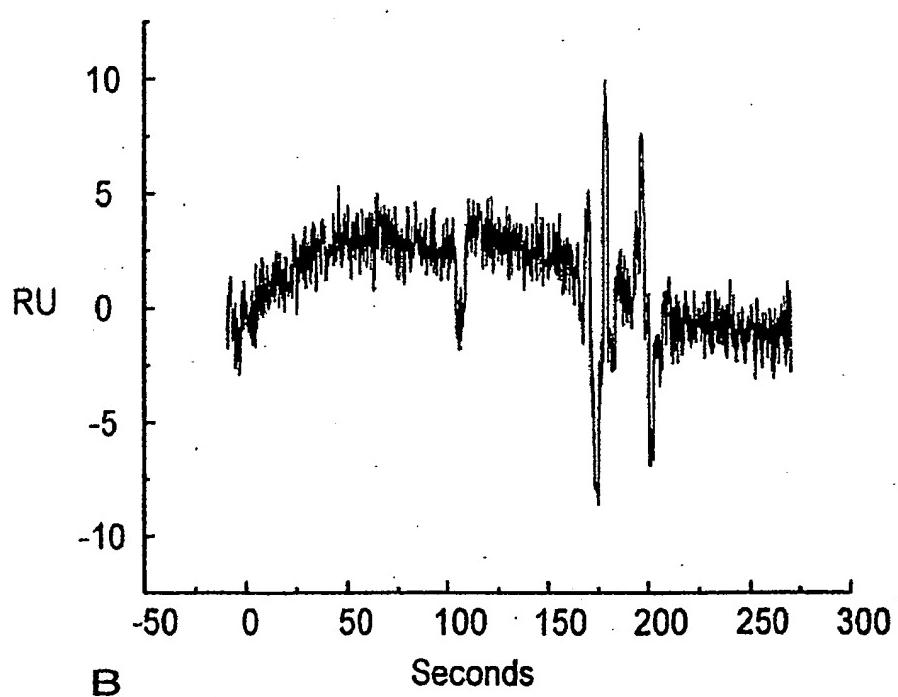
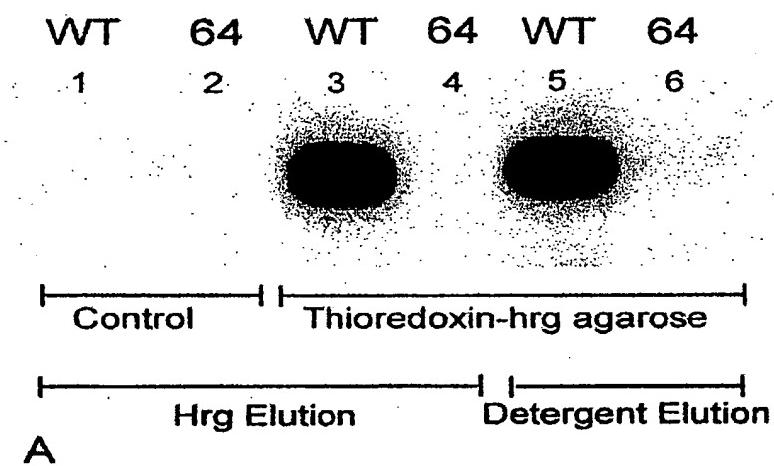


FIG. 10

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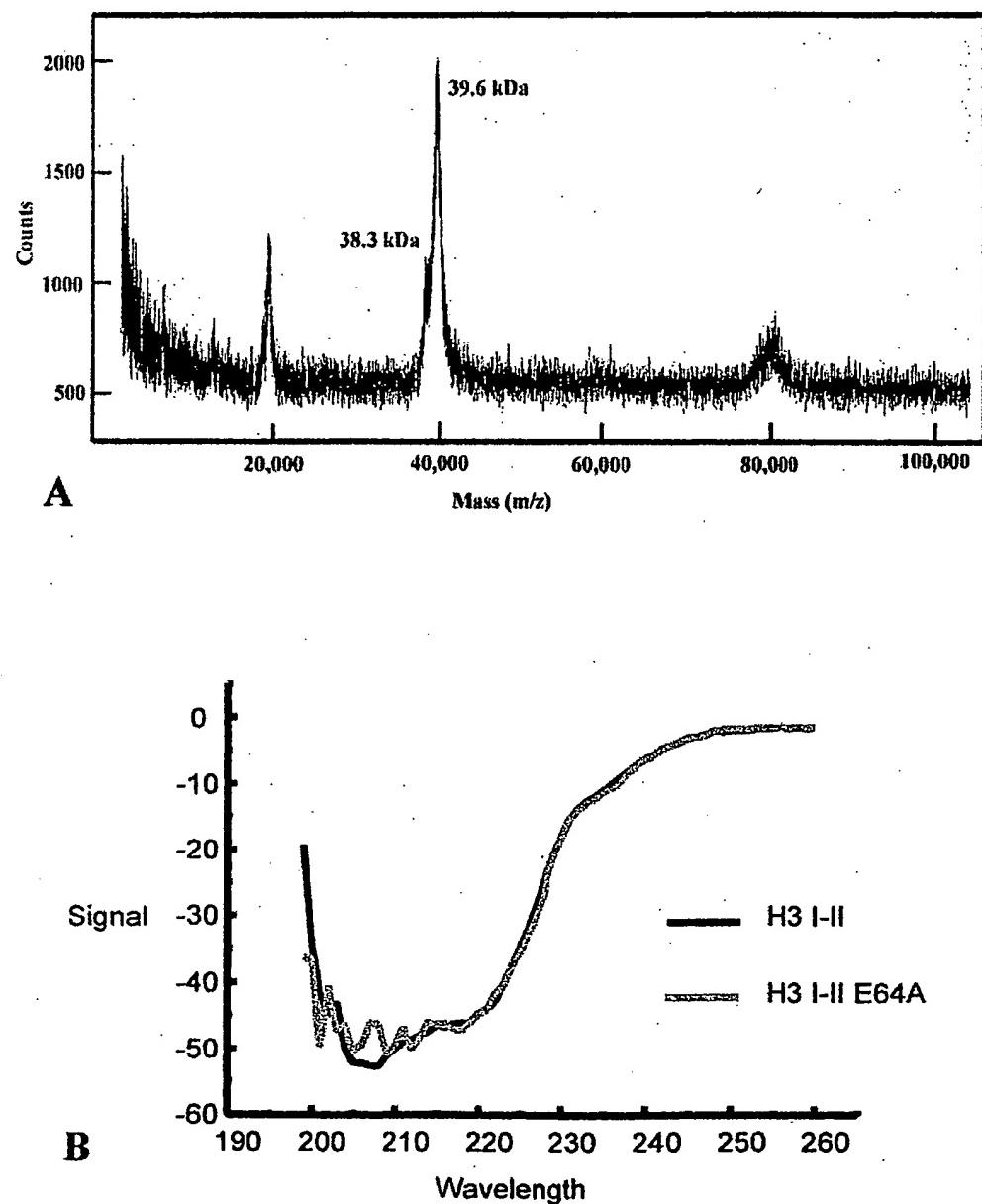


FIG. 11

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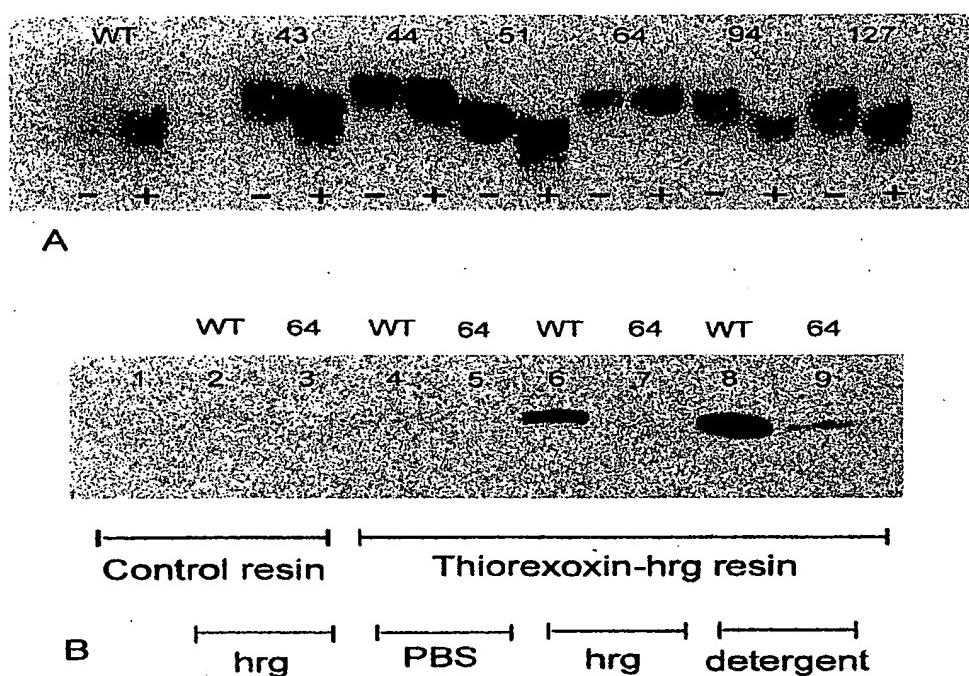
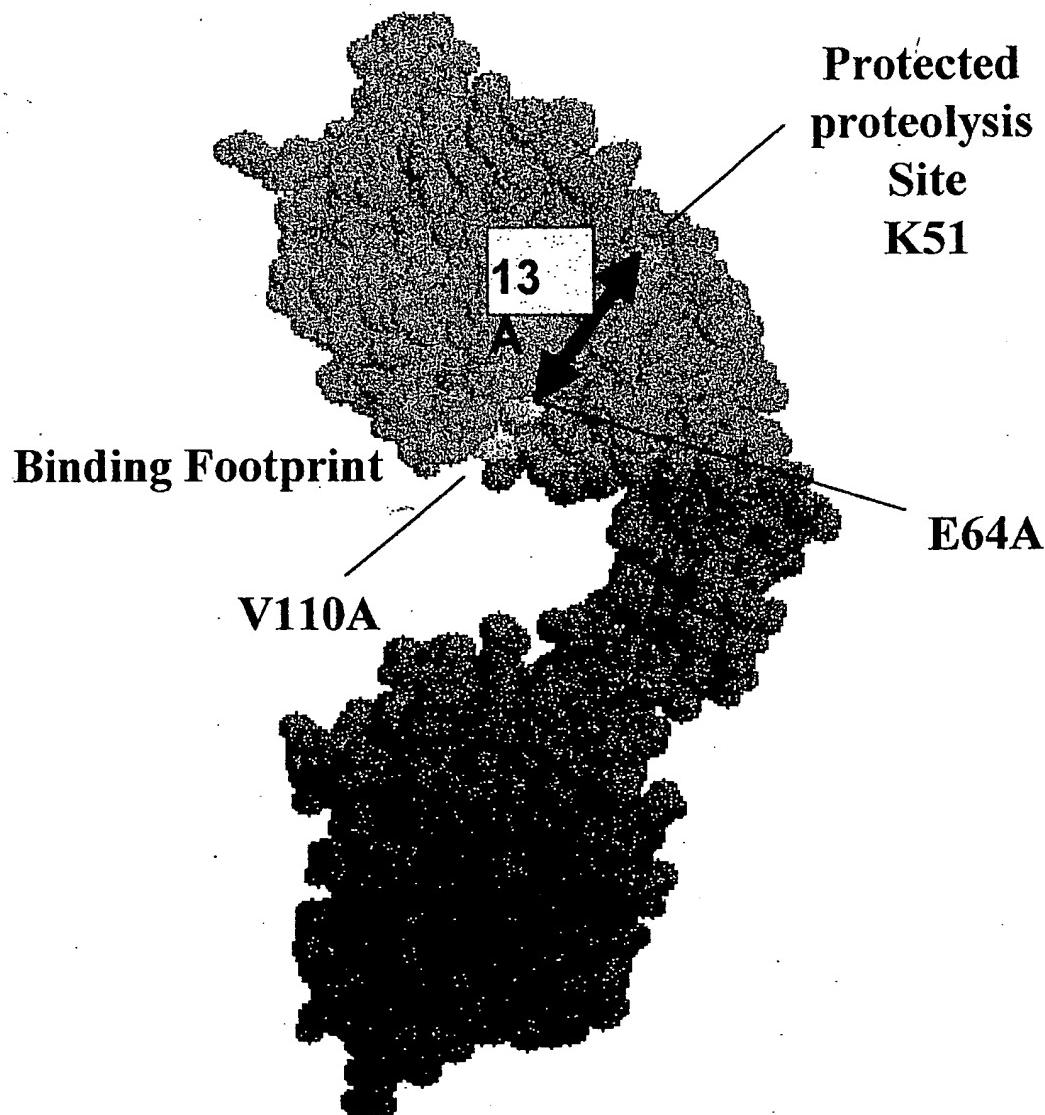
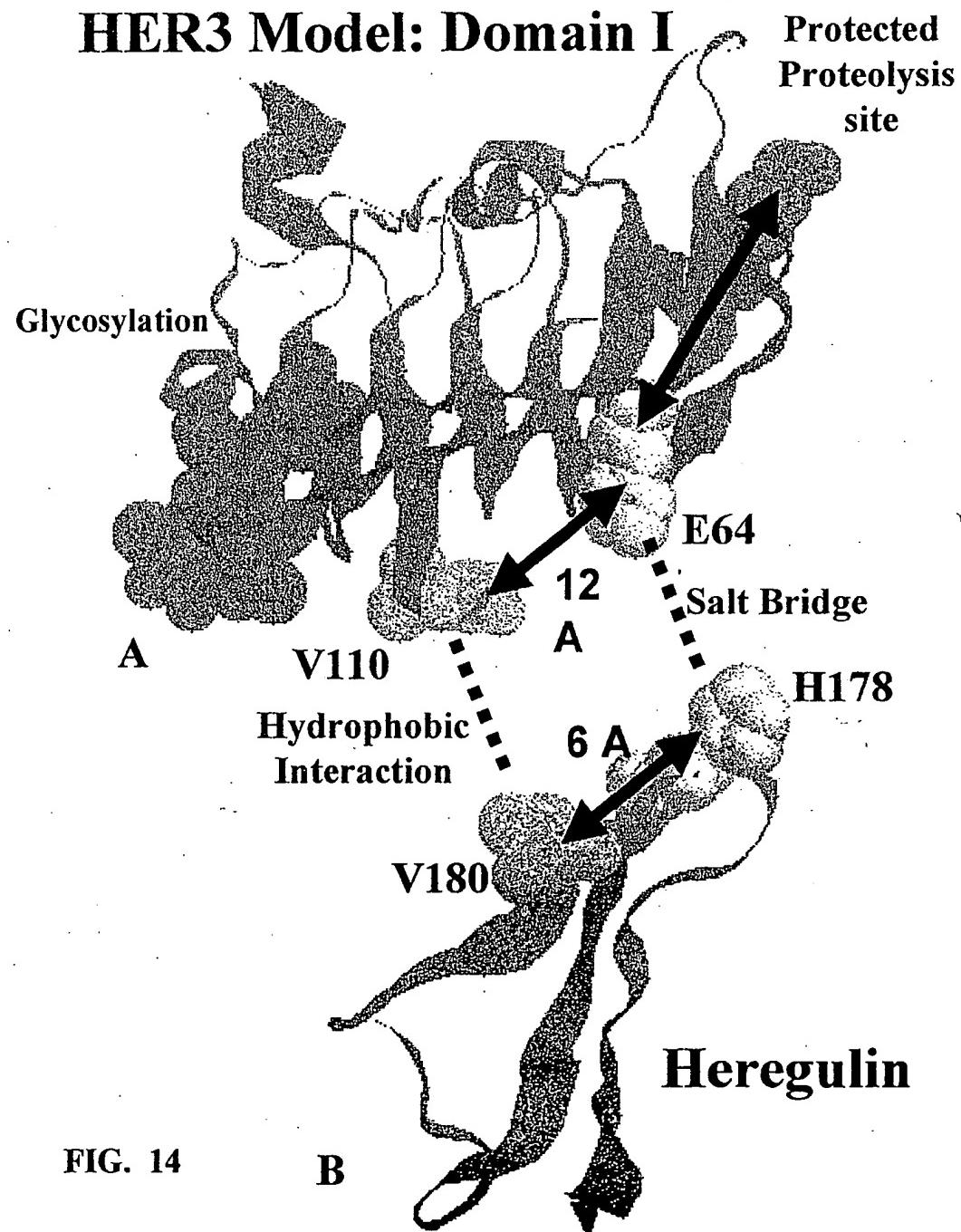


FIG. 12

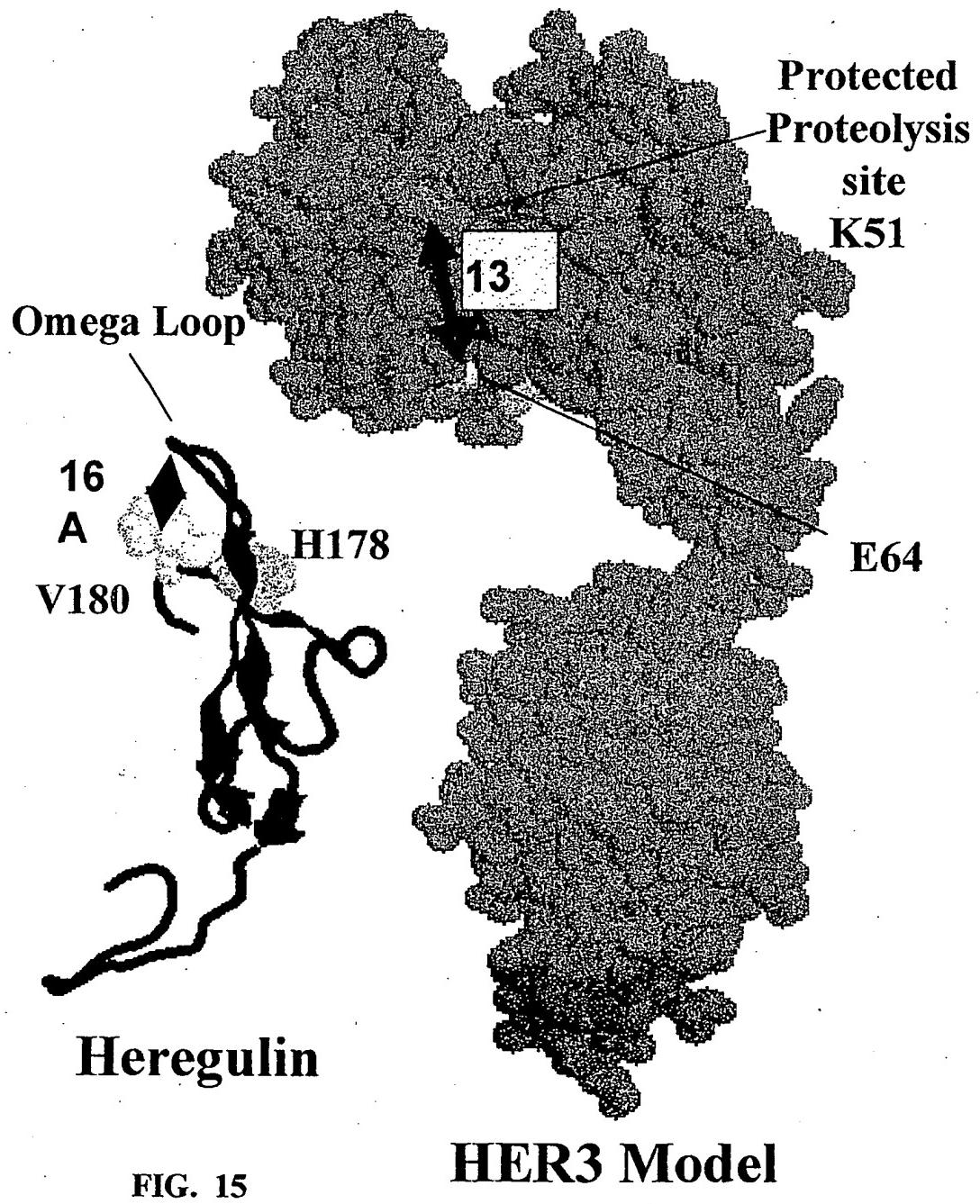
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**FIG. 13**

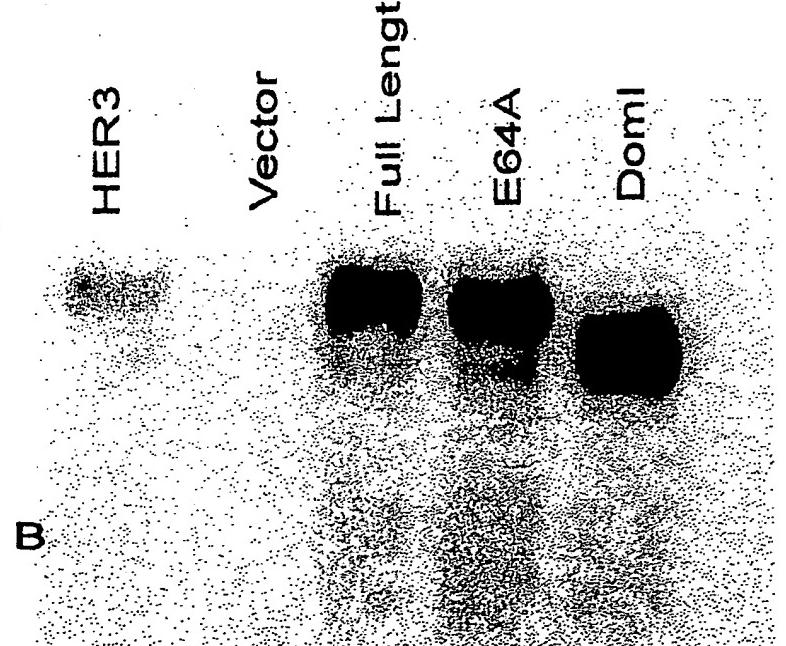
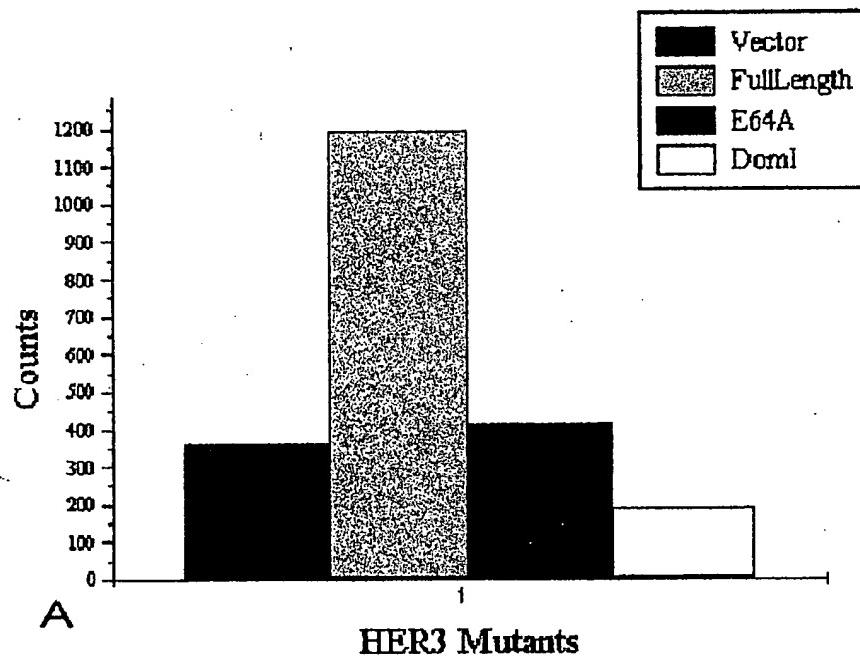
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**FIG. 16**

## SEQUENCE LISTING

&lt;110&gt; The Regents of the University of California et al.

<120> METHODS AND MATERIALS FOR CHARACTERIZING  
AND MODULATING INTERACTIONS BETWEEN HEREGULIN AND HER3

&lt;130&gt; 30448103WOU1

&lt;150&gt; 60/308,431

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Pro Arg Cys Pro Gln Pro Leu Val Tyr Asn Lys Leu Thr Phe Gln Leu			
260	265	270	
gaa ccc aat ccc cac acc aag tat cag tat gga gga gtt tgt gta gcc			864
Glu Pro Asn Pro His Thr Lys Tyr Gln Tyr Gly Gly Val Cys Val Ala			
275	280	285	
agc tgt ccc cat aac ttt gtg gtg gat caa aca tcc tgt gtc agg gcc			912
Ser Cys Pro His Asn Phe Val Val Asp Gln Thr Ser Cys Val Arg Ala			
290	295	300	
tgt cct cct gac aag atg gaa gta gat aaa aat ggg ctc aag atg tgt			960
Cys Pro Pro Asp Lys Met Glu Val Asp Lys Asn Gly Leu Lys Met Cys			

305	310	315	320
gag cct tgt ggg gga cta tgt ccc aaa gcc tgt gag gga aca ggc tct			
1008			
Glu	Pro	Cys	Gly
Gly	Leu	Cys	Pro
Lys	Ala	Cys	Glu
		Gly	Thr
			Gly
325		330	335
ggg agc cgc ttc cag act gtg gac tcg agc aac att gat gga ttt gtg			
1056			
Gly	Ser	Arg	Phe
Gln	Thr	Val	Asp
Ser	Ser	Asn	Ile
		Asp	Gly
340		345	350
aac tgc acc aag atc ctg ggc aac ctg gac ttt ctg atc acc ggc ctc			
1104			
Asn	Cys	Thr	Lys
Ile	Leu	Gly	Asn
Leu	Asp	Phe	Leu
		Ile	Thr
355		360	365
aat gga gac ccc tgg cac aag atc cct gcc ctg gac cca gag aag ctc			
1152			
Asn	Gly	Asp	Pro
Trp	His	Ile	Pro
		Ala	Leu
		Asp	Pro
		Glu	Lys
370		375	380
aat gtc ttc cgg aca gta cgg gag atc aca ggt tac ctg aac atc cag			
1200			
Asn	Val	Phe	Arg
		Thr	Val
		Arg	Glu
		Ile	Thr
		Gly	Tyr
		Leu	Asn
		Ile	Gln
385		390	395
tcc tgg ccg ccc cac atg cac aac ttc agt gtt ttt tcc aat ttg aca			
1248			
Ser	Trp	Pro	Pro
		His	Met
		Asn	Phe
		Ser	Val
		Phe	Ser
		Asn	Leu
		Leu	Thr
405		410	415
acc att gga ggc aga agc ctc tac aac cgg ggc ttc tca ttg ttg atc			
1296			
Thr	Ile	Gly	Gly
		Arg	Ser
		Leu	Tyr
		Asn	Arg
		Gly	Phe
		Ser	Leu
		Ile	Ile
420		425	430
atg aag aac ttg aat gtc aca tct ctg ggc ttc cga tcc ctg aag gaa			
1344			
Met	Lys	Asn	Leu
		Asn	Val
		Thr	Ser
		Leu	Gly
			Phe
			Arg
			Ser
			Leu
			Lys
435		440	445
att agt gct ggg cgt atc tat ata agt gcc aat agg cag ctc tgc tac			
1392			
Ile	Ser	Ala	Gly
		Arg	Ile
		Tyr	Ile
		Ser	Ala
		Asn	Arg
		Gln	Leu
		Cys	Tyr
450		455	460
cac cac tct ttg aac tgg acc aag gtg ctt cgg ggg cct acg gaa gag			
1440			
His	His	Ser	Leu
		Asn	Trp
		Thr	lys
		Val	Leu
		Arg	Gly
		Pro	Thr
		Glu	Glu
465		470	475
480			

cga cta gac atc aag cat aat cgg ccg cgc aga gac tgc gtg gca gag  
 1488  
 Arg Leu Asp Ile Lys His Asn Arg Pro Arg Arg Asp Cys Val Ala Glu  
 485                   490                   495

ggc aaa gtg tgt gac cca ctg tgc tcc tct ggg gga tgc tgg ggc cca  
 1536  
 Gly Lys Val Cys Asp Pro Leu Cys Ser Ser Gly Gly Cys Trp Gly Pro  
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ggc cct ggt cag tgc ttg tcc tgt cga aat tat agc cga gga ggt gtc  
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 Gly Pro Gly Gln Cys Leu Ser Cys Arg Asn Tyr Ser Arg Gly Gly Val  
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tgt gtg acc cac tgc aac ttt ctg aat ggg gag cct cga gaa ttt gcc  
 1632  
 Cys Val Thr His Cys Asn Phe Leu Asn Gly Glu Pro Arg Glu Phe Ala  
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 His Glu Ala Glu Cys Phe Ser Cys His Pro Glu Cys Gln Pro Met Gly  
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 1728  
 Gly Thr Ala Thr Cys Asn Gly Ser Gly Ser Asp Thr Cys Ala Gln Cys  
 565                   570                   575

gcc cat ttt cga gat ggg ccc cac tgt gtg agc agc tgc ccc cat gga  
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 Ala His Phe Arg Asp Gly Pro His Cys Val Ser Ser Cys Pro His Gly  
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 1824  
 Val Leu Gly Ala Lys Gly Pro Ile Tyr Lys Tyr Pro Asp Val Gln Asn  
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gaa tgt cgg ccc tgc cat gag aac tgc acc cag ggg tgt aaa gga cca  
 1872  
 Glu Cys Arg Pro Cys His Glu Asn Cys Thr Gln Gly Cys Lys Gly Pro  
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 1920  
 Glu Leu Gln Asp Cys Leu Gly Gln Thr Leu Val Leu Ile Gly Lys Thr  
 625                   630                   635                   640

cat ctg aca atg gct ttg aca gtg ata gca gga ttg gta gtg att ttc.  
 1968  
 His Leu Thr Met Ala Leu Thr Val Ile Ala Gly Leu Val Val Ile Phe

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2016		
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2064		
Asn Lys Arg Ala Met Arg Arg Tyr Leu Glu Arg Gly Glu Ser Ile Glu		
675	680	685
cct ctg gac ccc agt gag aag gct aac aaa gtc ttg gcc aga atc ttc		
2112		
Pro Leu Asp Pro Ser Glu Lys Ala Asn Lys Val Leu Ala Arg Ile Phe		
690	695	700
aaa gag aca gag cta agg aag ctt aaa gtg ctt ggc tcg ggt gtc ttt		
2160		
Lys Glu Thr Glu Leu Arg Lys Leu Lys Val Leu Gly Ser Gly Val Phe		
705	710	715
720		
gga act gtg cac aaa gga gtg tgg atc cct gag ggt gaa tca atc aag		
2208		
Gly Thr Val His Lys Gly Val Trp Ile Pro Glu Gly Glu Ser Ile Lys		
725	730	735
att cca gtc tgc att aaa gtc att gag gac aag agt gga cgg cag agt		
2256		
Ile Pro Val Cys Ile Lys Val Ile Glu Asp Lys Ser Gly Arg Gln Ser		
740	745	750
ttt caa gct gtg aca gat cat atg ctg gcc att ggc agc ctg gac cat		
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Phe Gln Ala Val Thr Asp His Met Leu Ala Ile Gly Ser Leu Asp His		
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850 855 860  
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2640  
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Glu Ser Ile His Phe Gly Lys Tyr Thr His Gln Ser Asp Val Trp Ser  
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Tyr Gly Val Thr Val Trp Glu Leu Met Thr Phe Gly Ala Glu Pro Tyr  
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Val Lys Cys Trp Met Ile Asp Glu Asn Ile Arg Pro Thr Phe Lys Glu  
945 950 955 960  
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Leu Ala Asn Glu Phe Thr Arg Met Ala Arg Asp Pro Pro Arg Tyr Leu  
965 970 975  
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2976  
Val Ile Lys Arg Glu Ser Gly Pro Gly Ile Ala Pro Gly Pro Glu Pro

980

985

990

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 His Gly Leu Thr Asn Lys Lys Leu Glu Glu Val Glu Leu Glu Pro Glu  
 995 1000 1005  
  
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 3072  
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 Thr Thr Leu Gly Ser Ala Leu Ser Leu Pro Val Gly Thr Leu Asn Arg  
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 Pro Arg Gly Ser Gln Ser Leu Leu Ser Pro Ser Ser Gly Tyr Met Pro  
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 Gly Ser Ser Glu Arg Cys Pro Arg Pro Val Ser Leu His Pro Met Pro  
 1075 1080 1085  
  
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 3312  
 Arg Gly Cys Leu Ala Ser Glu Ser Ser Glu Gly His Val Thr Gly Ser  
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 gag gct gag ctc cag gag aaa gtg tca atg tgt aga agc cgg agc agg  
 3360  
 Glu Ala Glu Leu Gln Glu Lys Val Ser Met Cys Arg Ser Arg Ser Arg  
 1105 1110 1115 1120  
  
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 Ser Arg Ser Pro Arg Pro Arg Gly Asp Ser Ala Tyr His Ser Gln Arg  
 1125 1130 1135  
  
 cac agt ctg ctg act cct gtt acc cca ctc tcc cca ccc ggg tta gag  
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 His Ser Leu Leu Thr Pro Val Thr Pro Leu Ser Pro Pro Gly Leu Glu  
 1140 1145 1150

gaa gag gat gtc aac ggt tat gtc atg cca gat aca cac ctc aaa ggt  
 3504  
 Glu Glu Asp Val Asn Gly Tyr Val Met Pro Asp Thr His Leu Lys Gly  
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 act ccc tcc tcc cgg gaa ggc acc ctt tct tca gtg ggt ctc agt tct  
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 Thr Pro Ser Ser Arg Glu Gly Thr Leu Ser Ser Val Gly Leu Ser Ser  
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 Val Leu Gly Thr Glu Glu Asp Glu Asp Glu Glu Tyr Glu Tyr Met  
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 aac cgg agg aga agg cac agt cca cct cat ccc cct agg cca agt tcc  
 3648  
 Asn Arg Arg Arg Arg His Ser Pro Pro His Pro Pro Arg Pro Ser Ser  
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 ctt gag gag ctg ggt tat gag tac atg gat gtg ggg tca gac ctc agt  
 3696  
 Leu Glu Glu Leu Gly Tyr Glu Tyr Met Asp Val Gly Ser Asp Leu Ser  
       1220                 1225                 1230  
  
 gcc tct ctg ggc agc aca cag agt tgc cca ctc cac cct gta ccc atc  
 3744  
 Ala Ser Leu Gly Ser Thr Gln Ser Cys Pro Leu His Pro Val Pro Ile  
       1235                 1240                 1245  
  
 atg ccc act gca ggc aca act cca gat gaa gac tat gaa tat atg aat  
 3792  
 Met Pro Thr Ala Gly Thr Thr Pro Asp Glu Asp Tyr Glu Tyr Met Asn  
       1250                 1255                 1260  
  
 cgg caa cga gat gga ggt ggt cct ggg ggt gat tat gca gcc atg ggg  
 3840  
 Arg Gln Arg Asp Gly Gly Pro Gly Gly Asp Tyr Ala Ala Met Gly  
       1265                 1270                 1275                 1280  
  
 gcc tgc cca gca tct gag caa ggg tat gaa gag atg aga gct ttt cag  
 3888  
 Ala Cys Pro Ala Ser Glu Gln Gly Tyr Glu Glu Met Arg Ala Phe Gln  
       1285                 1290                 1295  
  
 ggg cct gga cat cag gcc ccc cat gtc cat tat gcc cgc cta aaa act  
 3936  
 Gly Pro Gly His Gln Ala Pro His Val His Tyr Ala Arg Leu Lys Thr  
       1300                 1305                 1310  
  
 cta cgt agc tta gag gct aca gac tct gcc ttt gat aac cct gat tac  
 3984  
 Leu Arg Ser Leu Glu Ala Thr Asp Ser Ala Phe Asp Asn Pro Asp Tyr

1315

1320

1325

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tgg cat agc agg ctt ttc ccc aag gct aat gcc cag aga acg
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 Leu Asn Gly Leu Ser Val Thr Gly Asp Ala Glu Asn Gln Tyr Gln Thr  
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 Leu Tyr Lys Leu Tyr Glu Arg Cys Glu Val Val Met Gly Asn Leu Glu  
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 Arg Glu Val Thr Gly Tyr Val Leu Val Ala Met Asn Glu Phe Ser Thr  
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 Gly Lys Phe Ala Ile Phe Val Met Leu Asn Tyr Asn Thr Asn Ser Ser  
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 Gly Gly Val Tyr Ile Glu Lys Asn Asp Lys Leu Cys His Met Asp Thr  
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 Lys Asp Asn Gly Arg Ser Cys Pro Pro Cys His Glu Val Cys Lys Gly  
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 Arg Cys Trp Gly Pro Gly Ser Glu Asp Cys Gln Thr Leu Thr Lys Thr  
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 Thr Asp Cys Phe Ala Cys Arg His Phe Asn Asp Ser Gly Ala Cys Val  
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 Pro Arg Cys Pro Gln Pro Leu Val Tyr Asn Lys Leu Thr Phe Gln Leu  
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Cys Pro Pro Asp Lys Met Glu Val Asp Lys Asn Gly Leu Lys Met Cys  
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 355 360 365  
 Asn Gly Asp Pro Trp His Lys Ile Pro Ala Leu Asp Pro Glu Lys Leu  
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 Met Lys Asn Leu Asn Val Thr Ser Leu Gly Phe Arg Ser Leu Lys Glu  
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 Gly Pro Gly Gln Cys Leu Ser Cys Arg Asn Tyr Ser Arg Gly Gly Val  
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Ala His Ile Val Arg Leu Leu Gly Leu Cys Pro Gly Ser Ser Leu Gln		
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Gln Val Ala Asp Phe Gly Val Ala Asp Leu Leu Pro Pro Asp Asp Lys		
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Val Lys Cys Trp Met Ile Asp Glu Asn Ile Arg Pro Thr Phe Lys Glu		
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Leu Ala Asn Glu Phe Thr Arg Met Ala Arg Asp Pro Pro Arg Tyr Leu		
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Val Ile Lys Arg Glu Ser Gly Pro Gly Ile Ala Pro Gly Pro Glu Pro		
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His Gly Leu Thr Asn Lys Lys Leu Glu Glu Val Glu Leu Glu Pro Glu		
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Thr Thr Leu Gly Ser Ala Leu Ser Leu Pro Val Gly Thr Leu Asn Arg		
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Pro Arg Gly Ser Gln Ser Leu Leu Ser Pro Ser Ser Gly Tyr Met Pro		
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Met Asn Gln Gly Asn Leu Gly Gly Ser Cys Gln Glu Ser Ala Val Ser		
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Gly Ser Ser Glu Arg Cys Pro Arg Pro Val Ser Leu His Pro Met Pro		
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Arg Gly Cys Leu Ala Ser Glu Ser Ser Glu Gly His Val Thr Gly Ser		
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Glu Ala Glu Leu Gln Glu Lys Val Ser Met Cys Arg Ser Arg Ser Arg		
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Ser Arg Ser Pro Arg Pro Arg Gly Asp Ser Ala Tyr His Ser Gln Arg		
1125	1130	1135
His Ser Leu Leu Thr Pro Val Thr Pro Leu Ser Pro Pro Gly Leu Glu		
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 Glu Arg Gly Ser Gly Lys Lys Pro Glu Ser Ala Ala Gly Ser Gln Ser  
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 Pro Ala Leu Pro Pro Gln Leu Lys Glu Met Lys Ser Gln Glu Ser Ala  
 35 40 45  
 gca ggt tcc aaa cta gtc ctt cgg tgt gaa acc agt tct gaa tac tcc 192  
 Ala Gly Ser Lys Leu Val Leu Arg Cys Glu Thr Ser Ser Glu Tyr Ser  
 50 55 60  
 tct ctc aga ttc aag tgg ttc aag aat ggg aat gaa ttg aat cga aaa 240

Ser	Leu	Arg	Phe	Lys	Trp	Phe	Lys	Asn	Gly	Asn	Glu	Leu	Asn	Arg	Lys	
65					70			75			80					
aac	aaa	cca	caa	aat	atc	aag	ata	caa	aaa	aag	cca	ggg	aag	tca	gaa	288
Asn	Lys	Pro	Gln	Asn	Ile	Lys	Ile	Gln	Lys	Lys	Pro	Gly	Lys	Ser	Glu	
					85			90			95					
ctt	cgc	att	aac	aaa	gca	tca	ctg	gct	gat	tct	gga	gag	tat	atg	tgc	336
Leu	Arg	Ile	Asn	Lys	Ala	Ser	Leu	Ala	Asp	Ser	Gly	Glu	Tyr	Met	Cys	
					100			105			110					
aaa	gtg	atc	agc	aaa	tta	gga	aat	gac	agt	gcc	tct	gcc	aat	atc	acc	384
Lys	Val	Ile	Ser	Lys	Leu	Gly	Asn	Asp	Ser	Ala	Ser	Ala	Asn	Ile	Thr	
					115			120			125					
atc	gtg	gaa	tca	aac	gag	atc	atc	act	ggt	atg	cca	gcc	tca	act	gaa	432
Ile	Val	Glu	Ser	Asn	Glu	Ile	Ile	Thr	Gly	Met	Pro	Ala	Ser	Thr	Glu	
					130			135			140					
gga	gca	tat	gtg	tct	tca	gag	tct	ccc	att	aga	ata	tca	gta	tcc	aca	480
Gly	Ala	Tyr	Val	Ser	Ser	Glu	Ser	Pro	Ile	Arg	Ile	Ser	Val	Ser	Thr	
					145			150			155			160		
gaa	gga	gca	aat	act	tct	tca	tct	aca	tct	aca	tcc	acc	act	ggg	aca	528
Glu	Gly	Ala	Asn	Ser	Ser	Ser	Ser	Thr	Ser	Thr	Ser	Thr	Thr	Thr	Gly	Thr
					165			170			175					
agc	cat	ctt	gta	aaa	tgt	gcg	gag	aag	gag	aaa	act	ttc	tgt	gtg	aat	576
Ser	His	Leu	Val	Lys	Cys	Ala	Glu	Lys	Glu	Lys	Thr	Phe	Cys	Val	Asn	
					180			185			190					
gga	ggg	gag	tgc	ttc	atg	gtg	aaa	gac	ctt	tca	aac	ccc	tcg	aga	tac	624
Gly	Gly	Glu	Cys	Phe	Met	Val	Lys	Asp	Leu	Ser	Asn	Pro	Ser	Arg	Tyr	
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ttg	tgc	aag	tgc	cca	aat	gag	ttt	act	ggt	gat	cgc	tgc	caa	aac	tac	672
Leu	Cys	Lys	Cys	Pro	Asn	Glu	Phe	Thr	Gly	Asp	Arg	Cys	Gln	Asn	Tyr	
					210			215			220					
gta	atg	gcc	agc	ttc	tac	aag	cat	ctt	ggg	att	gaa	ttt	atg	gag	gca	720
Val	Met	Ala	Ser	Phe	Tyr	Lys	His	Leu	Gly	Ile	Glu	Phe	Met	Glu	Ala	
					225			230			235			240		
gag	gag	ctg	tac	cag	aag	aga	gtg	ctg	acc	ata	acc	ggc	atc	tgc	atc	768
Glu	Glu	Leu	Tyr	Gln	Lys	Arg	Val	Leu	Thr	Ile	Thr	Gly	Ile	Cys	Ile	
					245			250			255					
gcc	ctc	ctt	gtg	gtc	ggc	atc	atg	tgt	gtg	gtg	gcc	tac	tgc	aaa	acc	816
Ala	Leu	Leu	Val	Val	Gly	Ile	Met	Cys	Val	Val	Ala	Tyr	Cys	Lys	Thr	
					260			265			270					
aag	aaa	cag	cgg	aaa	aag	ctg	cat	gac	cgt	ctt	cgg	cag	agc	ctt	cgg	864
Lys	Lys	Gln	Arg	Lys	Lys	Leu	His	Asp	Arg	Leu	Arg	Gln	Ser	Leu	Arg	

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tct gaa cga aac aat atg atg aac att gcc aat ggg cct cac cat cct Ser Glu Arg Asn Asn Met Met Asn Ile Ala Asn Gly Pro His His Pro			912
290	295	300	
aac cca ccc ccc gag aat gtc cag ctg gtg aat caa tac gta tct aaa Asn Pro Pro Pro Glu Asn Val Gln Leu Val Asn Gln Tyr Val Ser Lys			960
305	310	315	320
aac gtc atc tcc agt gag cat att gtt gag aga gaa gca gag aca tcc 1008 Asn Val Ile Ser Ser Glu His Ile Val Glu Arg Glu Ala Glu Thr Ser			
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ttt tcc acc agt cac tat act tcc aca gcc cat cac tcc act act gtc 1056 Phe Ser Thr Ser His Tyr Thr Ser Thr Ala His His Ser Thr Thr Val			
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acc cag act cct agc cac agc tgg agc aac gga cac act gaa agc atc 1104 Thr Gln Thr Pro Ser His Ser Trp Ser Asn Gly His Thr Glu Ser Ile			
355	360	365	
ctt tcc gaa agc cac tct gta atc gtg atg tca tcc gta gaa aac agt 1152 Leu Ser Glu Ser His Ser Val Ile Val Met Ser Ser Val Glu Asn Ser			
370	375	380	
agg cac agc agc cca act ggg ggc cca aga gga cgt ctt aat ggc aca 1200 Arg His Ser Ser Pro Thr Gly Gly Pro Arg Gly Arg Leu Asn Gly Thr			
385	390	395	400
gga ggc cct cgt gaa tgt aac agc ttc ctc agg cat gcc aga gaa acc 1248 Gly Gly Pro Arg Glu Cys Asn Ser Phe Leu Arg His Ala Arg Glu Thr			
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cct gat tcc tac cga gac tct cct cat agt gaa agg tat gtg tca gcc 1296 Pro Asp Ser Tyr Arg Asp Ser Pro His Ser Glu Arg Tyr Val Ser Ala			
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tcc ccc aaa tcg ccc cct tcg gaa atg tct cca ccc gtg tcc agc atg 1392 Ser Pro Lys Ser Pro Pro Ser Glu Met Ser Pro Pro Val Ser Ser Met			

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acg gtg tcc atg cct tcc atg gcg gtc agc ccc ttc atg gaa gaa gag		
1440		
Thr Val Ser Met Pro Ser Met Ala Val Ser Pro Phe Met Glu Glu		
465	470	475
480		
aga cct cta ctt ctc gtg aca cca cca agg ctg cggt gag aag aag ttt		
1488		
Arg Pro Leu Leu Leu Val Thr Pro Pro Arg Leu Arg Glu Lys Lys Phe		
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gac cat cac cct cag cag ttc agc tcc ttc cac cac aac ccc gcg cat		
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Asp His His Pro Gln Gln Phe Ser Ser Phe His His Asn Pro Ala His		
500	505	510
gac agt aac agc ctc cct gct agc ccc ttg agg ata gtg gag gat gag		
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Asp Ser Asn Ser Leu Pro Ala Ser Pro Leu Arg Ile Val Glu Asp Glu		
515	520	525
gag tat gaa acg acc caa gag tac gag cca gcc caa gag cct gtt aag		
1632		
Glu Tyr Glu Thr Thr Gln Glu Tyr Glu Pro Ala Gln Glu Pro Val Lys		
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1680		
Lys Leu Ala Asn Ser Arg Arg Ala Lys Arg Thr Lys Pro Asn Gly His		
545	550	555
560		
att gct aac aga ttg gaa gtg gac agc aac aca agc tcc cag agc agt		
1728		
Ile Ala Asn Arg Leu Glu Val Asp Ser Asn Thr Ser Ser Gln Ser Ser		
565	570	575
aac tca gag agt gaa aca gaa gat gaa aga gta ggt gaa gat acg cct		
1776		
Asn Ser Glu Ser Glu Thr Glu Asp Glu Arg Val Gly Glu Asp Thr Pro		
580	585	590
590		
ttc ctg ggc ata cag aac ccc ctg gca gcc agt ctt gag gca aca cct		
1824		
Phe Leu Gly Ile Gln Asn Pro Leu Ala Ala Ser Leu Glu Ala Thr Pro		
595	600	605
605		
gcc ttc cgc ctg gct gac agc agg act aac cca gca ggc cgc ttc tcg		
1872		
Ala Phe Arg Leu Ala Asp Ser Arg Thr Asn Pro Ala Gly Arg Phe Ser		
610	615	620

aca cag gaa gaa atc cag gcc agg ctg tct agt gta att gct aac caa  
 1920  
 Thr Gln Glu Glu Ile Gln Ala Arg Leu Ser Ser Val Ile Ala Asn Gln  
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 gac cct att gct gta  
 1935  
 Asp Pro Ile Ala Val  
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 Pro Ala Leu Pro Pro Gln Leu Lys Glu Met Lys Ser Gln Glu Ser Ala  
 35               40                   45  
 Ala Gly Ser Lys Leu Val Leu Arg Cys Glu Thr Ser Ser Glu Tyr Ser  
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 Ser Leu Arg Phe Lys Trp Phe Lys Asn Gly Asn Glu Leu Asn Arg Lys  
 65               70                   75                   80  
 Asn Lys Pro Gln Asn Ile Lys Ile Gln Lys Lys Pro Gly Lys Ser Glu  
 85               90                   95  
 Leu Arg Ile Asn Lys Ala Ser Leu Ala Asp Ser Gly Glu Tyr Met Cys  
 100              105               110  
 Lys Val Ile Ser Lys Leu Gly Asn Asp Ser Ala Ser Ala Asn Ile Thr  
 115              120               125  
 Ile Val Glu Ser Asn Glu Ile Ile Thr Gly Met Pro Ala Ser Thr Glu  
 130              135               140  
 Gly Ala Tyr Val Ser Ser Glu Ser Pro Ile Arg Ile Ser Val Ser Thr  
 145              150               155                   160  
 Glu Gly Ala Asn Thr Ser Ser Ser Thr Ser Thr Ser Thr Thr Gly Thr  
 165              170               175  
 Ser His Leu Val Lys Cys Ala Glu Lys Glu Lys Thr Phe Cys Val Asn  
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 Gly Gly Glu Cys Phe Met Val Lys Asp Leu Ser Asn Pro Ser Arg Tyr  
 195              200               205  
 Leu Cys Lys Cys Pro Asn Glu Phe Thr Gly Asp Arg Cys Gln Asn Tyr  
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 Val Met Ala Ser Phe Tyr Lys His Leu Gly Ile Glu Phe Met Glu Ala  
 225              230               235                   240  
 Glu Glu Leu Tyr Gln Lys Arg Val Leu Thr Ile Thr Gly Ile Cys Ile  
 245              250               255  
 Ala Leu Leu Val Val Gly Ile Met Cys Val Val Ala Tyr Cys Lys Thr  
 260              265               270  
 Lys Lys Gln Arg Lys Lys Leu His Asp Arg Leu Arg Gln Ser Leu Arg  
 275              280               285

Ser Glu Arg Asn Asn Met Met Asn Ile Ala Asn Gly Pro His His Pro  
 290 295 300  
 Asn Pro Pro Pro Glu Asn Val Gln Leu Val Asn Gln Tyr Val Ser Lys  
 305 310 315 320  
 Asn Val Ile Ser Ser Glu His Ile Val Glu Arg Glu Ala Glu Thr Ser  
 325 330 335  
 Phe Ser Thr Ser His Tyr Thr Ser Thr Ala His His Ser Thr Thr Val  
 340 345 350  
 Thr Gln Thr Pro Ser His Ser Trp Ser Asn Gly His Thr Glu Ser Ile  
 355 360 365  
 Leu Ser Glu Ser His Ser Val Ile Val Met Ser Ser Val Glu Asn Ser  
 370 375 380  
 Arg His Ser Ser Pro Thr Gly Gly Pro Arg Gly Arg Leu Asn Gly Thr  
 385 390 395 400  
 Gly Gly Pro Arg Glu Cys Asn Ser Phe Leu Arg His Ala Arg Glu Thr  
 405 410 415  
 Pro Asp Ser Tyr Arg Asp Ser Pro His Ser Glu Arg Tyr Val Ser Ala  
 420 425 430  
 Met Thr Thr Pro Ala Arg Met Ser Pro Val Asp Phe His Thr Pro Ser  
 435 440 445  
 Ser Pro Lys Ser Pro Pro Ser Glu Met Ser Pro Pro Val Ser Ser Met  
 450 455 460  
 Thr Val Ser Met Pro Ser Met Ala Val Ser Pro Phe Met Glu Glu Glu  
 465 470 475 480  
 Arg Pro Leu Leu Leu Val Thr Pro Pro Arg Leu Arg Glu Lys Lys Phe  
 485 490 495  
 Asp His His Pro Gln Gln Phe Ser Ser Phe His His Asn Pro Ala His  
 500 505 510  
 Asp Ser Asn Ser Leu Pro Ala Ser Pro Leu Arg Ile Val Glu Asp Glu  
 515 520 525  
 Glu Tyr Glu Thr Thr Gln Glu Tyr Glu Pro Ala Gln Glu Pro Val Lys  
 530 535 540  
 Lys Leu Ala Asn Ser Arg Arg Ala Lys Arg Thr Lys Pro Asn Gly His  
 545 550 555 560  
 Ile Ala Asn Arg Leu Glu Val Asp Ser Asn Thr Ser Ser Gln Ser Ser  
 565 570 575  
 Asn Ser Glu Ser Glu Thr Glu Asp Glu Arg Val Gly Glu Asp Thr Pro  
 580 585 590  
 Phe Leu Gly Ile Gln Asn Pro Leu Ala Ala Ser Leu Glu Ala Thr Pro  
 595 600 605  
 Ala Phe Arg Leu Ala Asp Ser Arg Thr Asn Pro Ala Gly Arg Phe Ser  
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 Asp Pro Ile Ala Val  
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His	Pro	Cys	Ser	Pro	Met	Cys	Lys	Gly	Ser	Arg	Cys	Trp	Gly	Glu	Ser	
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Ser	Glu	Asp	Cys	Gln	Ser	Leu	Thr	Arg	Thr	Val	Cys	Ala	Gly	Gly	Cys	
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Ala	Arg	Cys	Lys	Gly	Pro	Leu	Pro	Thr	Asp	Cys	Cys	His	Glu	Gln	Cys	
225															240	
gct	gcc	ggc	tgc	acg	ggc	ccc	aag	cac	tct	gac	tgc	ctg	gcc	tgc	ctc	768
Ala	Ala	Gly	Cys	Thr	Gly	Pro	Lys	His	Ser	Asp	Cys	Leu	Ala	Cys	Leu	
245															255	
cac	ttc	aac	cac	agt	ggc	atc	tgt	gag	ctg	cac	tgc	cca	gcc	ctg	gtc	816
His	Phe	Asn	His	Ser	Gly	Ile	Cys	Glu	Leu	His	Cys	Pro	Ala	Leu	Val	
260															270	
acc	tac	aac	aca	gac	acg	ttt	gag	tcc	atg	ccc	aat	ccc	gag	ggc	cgg	864
Thr	Tyr	Asn	Thr	Asp	Thr	Phe	Glu	Ser	Met	Pro	Asn	Pro	Glu	Gly	Arg	
275															285	
tat	aca	ttc	ggc	gcc	agc	tgt	gtg	act	gcc	tgt	ccc	tac	aac	tac	ctt	912
Tyr	Thr	Phe	Gly	Ala	Ser	Cys	Val	Thr	Ala	Cys	Pro	Tyr	Asn	Tyr	Leu	
290															300	
tct	acg	gac	gtg	gga	tcc	tgc	acc	ctc	gtc	tgc	ccc	ctg	cac	aac	caa	960
Ser	Thr	Asp	Val	Gly	Ser	Cys	Thr	Leu	Val	Cys	Pro	Leu	His	Asn	Gln	
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gag	gtg	aca	gca	gag	gat	gga	aca	cag	cgg	tgt	gag	aag	tgc	agc	aag	
1008																
Glu	Val	Thr	Ala	Glu	Asp	Gly	Thr	Gln	Arg	Cys	Glu	Lys	Cys	Ser	Lys	
325															335	
ccc	tgt	gcc	cga	gtg	tgc	tat	ggt	ctg	ggc	atg	gag	cac	ttg	cga	gag	
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Pro	Cys	Ala	Arg	Val	Cys	Tyr	Gly	Leu	Gly	Met	Glu	His	Leu	Arg	Glu	
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1104																
Val	Arg	Ala	Val	Thr	Ser	Ala	Asn	Ile	Gln	Glu	Phe	Ala	Gly	Cys	Lys	
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aag	atc	ttt	ggg	agc	ctg	gca	ttt	ctg	ccg	gag	agc	ttt	gat	ggg	gac	
1152																
Lys	Ile	Phe	Gly	Ser	Leu	Ala	Phe	Leu	Pro	Glu	Ser	Phe	Asp	Gly	Asp	
370															380	

cca gcc tcc aac act gcc ccg ctc cag cca gag cag ctc caa gtg ttt  
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 Pro Ala Ser Asn Thr Ala Pro Leu Gln Pro Glu Gln Leu Gln Val Phe  
 385 390 395 400  
  
 gag act ctg gaa gag atc aca ggt tac cta tac atc tca gca tgg ccg  
 1248  
 Glu Thr Leu Glu Glu Ile Thr Gly Tyr Leu Tyr Ile Ser Ala Trp Pro  
 405 410 415  
  
 gac agc ctg cct gac ctc agc gtc ttc cag aac ctg caa gta atc cgg  
 1296  
 Asp Ser Leu Pro Asp Leu Ser Val Phe Gln Asn Leu Gln Val Ile Arg  
 420 425 430  
  
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 Gly Arg Ile Leu His Asn Gly Ala Tyr Ser Leu Thr Leu Gln Gly Leu  
 435 440 445  
  
 ggc atc agc tgg ctg ggg ctg cgc tca ctg agg gaa ctg ggc agt gga  
 1392  
 Gly Ile Ser Trp Leu Gly Leu Arg Ser Leu Arg Glu Leu Gly Ser Gly  
 450 455 460  
  
 ctg gcc ctc atc cac cat aac acc cac ctc tgc ttc gtg cac acg gtg  
 1440  
 Leu Ala Leu Ile His His Asn Thr His Leu Cys Phe Val His Thr Val  
 465 470 475 480  
  
 ccc tgg gac cag ctc ttt cgg aac ccg cac caa gct ctg ctc cac act  
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 Pro Trp Asp Gln Leu Phe Arg Asn Pro His Gln Ala Leu Leu His Thr  
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 gcc aac cgg cca gag gac gag tgt gtg ggc gag ggc ctg gcc tgc cac  
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 1584  
 Gln Leu Cys Ala Arg Gly His Cys Trp Gly Pro Gly Pro Thr Gln Cys  
 515 520 525  
  
 gtc aac tgc agc cag ttc ctt cgg ggc cag gag tgc gtg gag gaa tgc  
 1632  
 Val Asn Cys Ser Gln Phe Leu Arg Gly Gln Glu Cys Val Glu Glu Cys  
 530 535 540  
  
 cga gta ctg cag ggg ctc ccc agg gag tat gtg aat gcc agg cac tgt  
 1680  
 Arg Val Leu Gln Gly Leu Pro Arg Glu Tyr Val Asn Ala Arg His Cys

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ttt gga ccg gag gct gac cag cgt tgt gtg gcc tgt gcc cac tat aag gac 1776 Phe Gly Pro Glu Ala Asp Gln Cys Val Ala Cys Ala His Tyr Lys Asp 580                       585                       590			
cct ccc ttc tgc gtg gcc cgc tgc ccc agc ggt gtg aaa cct gac ctc 1824 Pro Pro Phe Cys Val Ala Arg Cys Pro Ser Gly Val Lys Pro Asp Leu 595                       600                       605			
tcc tac atg ccc atc tgg aag ttt cca gat gag gag ggc gca tgc cag 1872 Ser Tyr Met Pro Ile Trp Lys Phe Pro Asp Glu Glu Gly Ala Cys Gln 610                       615                       620			
cct tgc ccc atc aac tgc acc cac tcc tgt gtg gac ctg gat gac aag 1920 Pro Cys Pro Ile Asn Cys Thr His Ser Cys Val Asp Leu Asp Asp Lys 625                       630                       635                       640			
ggc tgc ccc gcc gag cag aga gcc agc cct ctg acg tcc atc gtc tct 1968 Gly Cys Pro Ala Glu Gln Arg Ala Ser Pro Leu Thr Ser Ile Val Ser 645                       650                       655			
gcg gtg gtt ggc att ctg ctg gtc gtg gtc ttg ggg gtg gtc ttt ggg 2016 Ala Val Val Gly Ile Leu Leu Val Val Val Leu Gly Val Val Phe Gly 660                       665                       670			
atc ctc atc aag cga cgg cag cag aag atc cgg aag tac acg atg cgg 2064 Ile Leu Ile Lys Arg Arg Gln Gln Lys Ile Arg Lys Tyr Thr Met Arg 675                       680                       685			
aga ctg ctg cag gaa acg gag ctg gtg gag ccc ctg aca cct agc gga 2112 Arg Leu Leu Gln Glu Thr Glu Leu Val Glu Pro Leu Thr Pro Ser Gly 690                       695                       700			
gcg atg ccc aac cag gcg cag atg cgg atc ctg aaa gag acg gag ctg 2160 Ala Met Pro Asn Gln Ala Gln Met Arg Ile Leu Lys Glu Thr Glu Leu 705                       710                       715                       720			

agg aag gtg aag gtg ctt gga tct ggc gct ttt ggc aca gtc tac aag  
2208

Arg Lys Val Lys Val Leu Gly Ser Gly Ala Phe Gly Thr Val Tyr Lys  
725                   730                   735

ggc atc tgg atc cct gat ggg gag aat gtg aaa att cca gtg gcc atc  
2256

Gly Ile Trp Ile Pro Asp Gly Glu Asn Val Lys Ile Pro Val Ala Ile  
740                   745                   750

aaa gtg ttg agg gaa aac aca tcc ccc aaa gcc aac aaa gaa atc tta  
2304

Lys Val Leu Arg Glu Asn Thr Ser Pro Lys Ala Asn Lys Glu Ile Leu  
755                   760                   765

gac gaa gca tac gtg atg gct ggt gtg ggc tcc cca tat gtc tcc cgc  
2352

Asp Glu Ala Tyr Val Met Ala Gly Val Gly Ser Pro Tyr Val Ser Arg  
770                   775                   780

ctt ctg ggc atc tgc ctg aca tcc acg gtg cag ctg gtg aca cag ctt  
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Leu Leu Gly Ile Cys Leu Thr Ser Thr Val Gln Leu Val Thr Gln Leu  
785                   790                   795                   800

atg ccc tat ggc tgc ctc tta gac cat gtc cgg gaa aac cgc gga cgc  
2448

Met Pro Tyr Gly Cys Leu Leu Asp His Val Arg Glu Asn Arg Gly Arg  
805                   810                   815

ctg ggc tcc cag gac ctg aac tgg tgt atg cag att gcc aag ggg  
2496

Leu Gly Ser Gln Asp Leu Leu Asn Trp Cys Met Gln Ile Ala Lys Gly  
820                   825                   830

atg agc tac ctg gag gat gtg cgg ctc gta cac agg gac ttg gcc gct  
2544

Met Ser Tyr Leu Glu Asp Val Arg Leu Val His Arg Asp Leu Ala Ala  
835                   840                   845

cgg aac gtg ctg gtc aag agt ccc aac cat gtc aaa att aca gac ttc  
2592

Arg Asn Val Leu Val Lys Ser Pro Asn His Val Lys Ile Thr Asp Phe  
850                   855                   860

ggg ctg gct cgg ctg gac att gac gag aca gag tac cat gca gat  
2640

Gly Leu Ala Arg Leu Leu Asp Ile Asp Glu Thr Glu Tyr His Ala Asp  
865                   870                   875                   880

ggg ggc aag gtg ccc atc aag tgg atg gcg ctg gag tcc att ctc cgc  
2688

Gly Gly Lys Val Pro Ile Lys Trp Met Ala Leu Glu Ser Ile Leu Arg

885	890	895
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2736		
Arg Arg Phe Thr His Gln Ser Asp Val Trp Ser Tyr Gly Val Thr Val		
900	905	910
tgg gag ctg atg act ttt ggg gcc aaa cct tac gat ggg atc cca gcc		
2784		
Trp Glu Leu Met Thr Phe Gly Ala Lys Pro Tyr Asp Gly Ile Pro Ala		
915	920	925
cg <del>g</del> gag atc cct gac ctg ctg gaa aag ggg gag cg <del>g</del> ctg ccc cag ccc		
2832		
Arg Glu Ile Pro Asp Leu Leu Glu Lys Gly Glu Arg Leu Pro Gln Pro		
930	935	940
ccc atc tgc acc att gat gtc tac atg atc atg gtc aaa tgt tgg atg		
2880		
Pro Ile Cys Thr Ile Asp Val Tyr Met Ile Met Val Lys Cys Trp Met		
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1225

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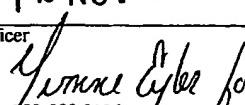
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**INTERNATIONAL SEARCH REPORT**

International application No.

PCT/US02/23963

<b>A. CLASSIFICATION OF SUBJECT MATTER</b>																	
IPC(7) : C07K 14/00; G01N 33/567; A61K 38/00 US CL : 530/350; 435/7.21; 514/2																	
According to International Patent Classification (IPC) or to both national classification and IPC																	
<b>B. FIELDS SEARCHED</b>																	
Minimum documentation searched (classification system followed by classification symbols) U.S. : 530/350; 435/7.21; 514/2																	
Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched																	
Electronic data base consulted during the international search (name of data base and, where practicable, search terms used) Please See Continuation Sheet																	
<b>C. DOCUMENTS CONSIDERED TO BE RELEVANT</b>																	
Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.															
Y	US 5,183844 A (KRAUS et al.) 2 February 1993 (02.02.1993), figure 5 and column 9, lines 23-42.	1, 2, 16-20															
Y	US 5,804,396 A (PLOWMAN) 8 September 1998 (08.09.1998), column 9, lines 28-47.	1, 2, 16-20															
<input type="checkbox"/> Further documents are listed in the continuation of Box C.		<input type="checkbox"/> See patent family annex.															
<p>Special categories of cited documents:</p> <table> <tr> <td>"A"</td> <td>"T"</td> <td>later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention</td> </tr> <tr> <td>"E"</td> <td>"X"</td> <td>document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone</td> </tr> <tr> <td>"L"</td> <td>"Y"</td> <td>document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art</td> </tr> <tr> <td>"O"</td> <td>"&amp;"</td> <td>document member of the same patent family</td> </tr> <tr> <td>"P"</td> <td></td> <td></td> </tr> </table>			"A"	"T"	later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention	"E"	"X"	document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone	"L"	"Y"	document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art	"O"	"&"	document member of the same patent family	"P"		
"A"	"T"	later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention															
"E"	"X"	document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone															
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"O"	"&"	document member of the same patent family															
"P"																	
Date of the actual completion of the international search <b>18 September 2002 (18.09.2002)</b>	Date of mailing of the international search report <b>12 NOV 2002</b>																
Name and mailing address of the ISA/US Commissioner of Patents and Trademarks Box PCT Washington, D.C. 20231 Facsimile No. (703)305-3230	Authorized officer  Janet Andres Telephone No. 703-308-0196																

**INTERNATIONAL SEARCH REPORT**

PCT/US02/23963

**Continuation of Item 4 of the first sheet:**

The title is more than seven words long. New title:

MODULATION OF HEREGULIN AND HER3 INTERACTION

**Continuation of B. FIELDS SEARCHED Item 3:**

STN, EAST

search terms: HER3, soluble receptor, ligand binding

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